BRINGING ADVANCED THERAPY MEDICINAL PRODUCTS (ATMPs) TO THE CLINIC AND BEYOND: HOW TO ENSURE THE SUSTAINABLE AND AFFORDABLE INTRODUCTION OF ATMPs INTO HEALTHCARE

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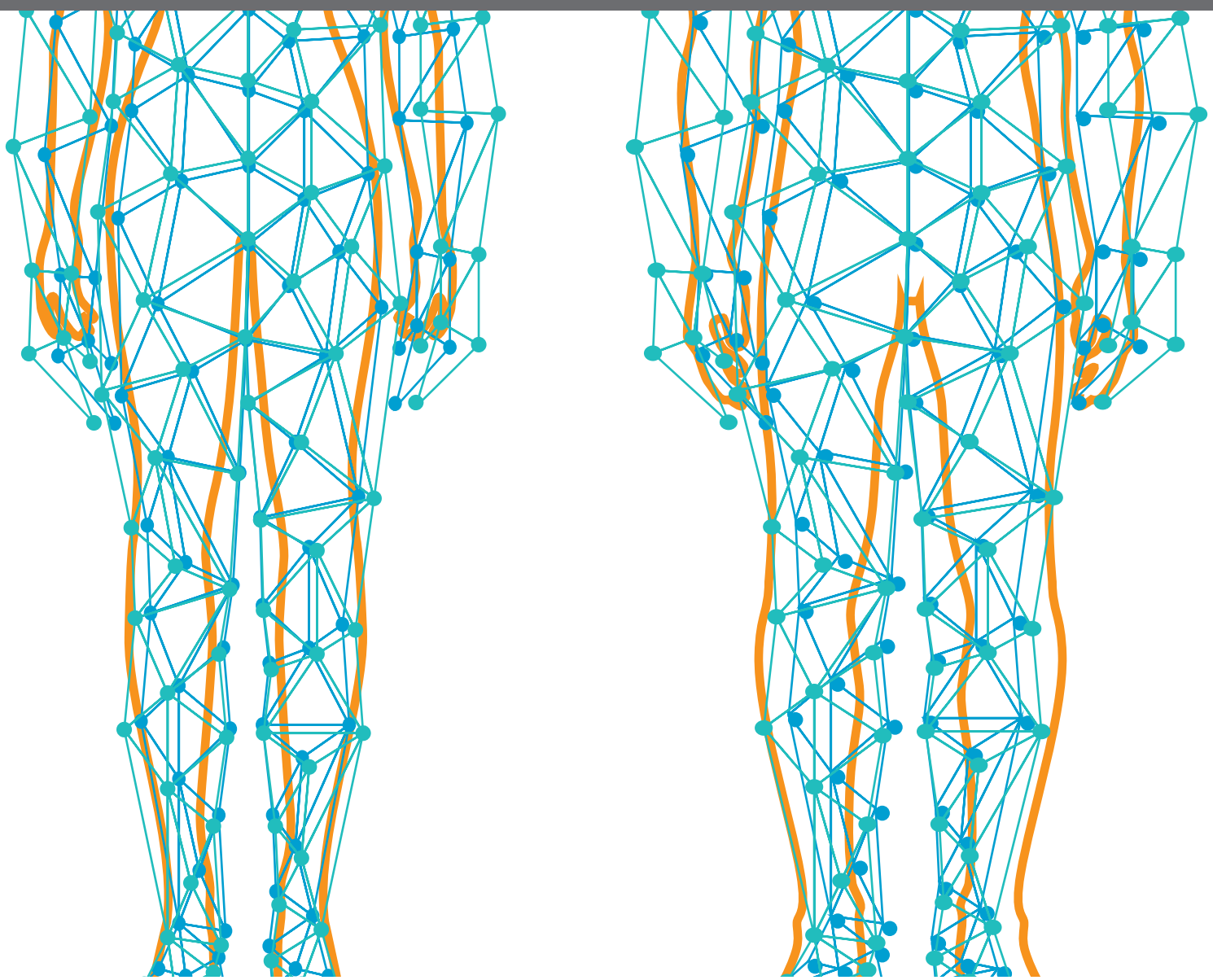
PUBLISHED IN: Frontiers in Medicine



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ISSN 1664-8714
ISBN 978-2-83250-780-3
DOI 10.3389/978-2-83250-780-3

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Citation: Volk, H.-D., Griscelli, A. B., Battaglia, M., Neves, N. M., eds. (2022). Bringing Advanced Therapy Medicinal Products (ATMPs) to the Clinic and Beyond: How to Ensure the Sustainable and Affordable Introduction of ATMPs Into Healthcare. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-780-3

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frontiers in Medicine

PERSPECTIVE
published: 13 August 2021
doi: 10.3389/fmed.2021.712917

From Single Batch to Mass Production–Automated Platform Design Concept for a Phase II Clinical Trial Tissue Engineered Cartilage Product

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OPEN ACCESS

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: 21 May 2021
Accepted: 19 July 2021
Published: 13 August 2021

Citation:
Haeusner S, Herbst L, Bittorf P, Schwarz T, Henze C, Mauermann M, Ochs J, Schmitt R, Blache U, Wixmerten A, Miot S, Martin I and Pullig O (2021) From Single Batch to Mass Production–Automated Platform Design Concept for a Phase II Clinical Trial Tissue Engineered Cartilage Product. Front. Med. 8:712917. doi: 10.3389/fmed.2021.712917

Advanced Therapy Medicinal Products (ATMP) provide promising treatment options particularly for unmet clinical needs, such as progressive and chronic diseases where currently no satisfying treatment exists. Especially from the ATMP subclass of Tissue Engineered Products (TEPs), only a few have yet been translated from an academic setting to clinic and beyond. A reason for low numbers of TEPs in current clinical trials and one main key hurdle for TEPs is the cost and labor-intensive manufacturing process. Manual production steps require experienced personnel, are challenging to standardize and to scale up. Automated manufacturing has the potential to overcome these challenges, toward an increasing cost-effectiveness. One major obstacle for automation is the control and risk prevention of cross contaminations, especially when handling parallel production lines of different patient material. These critical steps necessitate validated effective and efficient cleaning procedures in an automated system. In this perspective, possible technologies, concepts and solutions to existing ATMP manufacturing hurdles are discussed on the example of a late clinical phase II trial TEP. In compliance to Good Manufacturing Practice (GMP) guidelines, we propose a dual arm robot based isolator approach. Our novel concept enables complete process automation for adherent cell culture, and the translation of all manual process steps with standard laboratory equipment. Moreover, we discuss novel solutions for automated cleaning, without the need for human intervention. Consequently, our automation concept offers the unique chance to scale up production while becoming more cost-effective, which will ultimately increase TEP availability to a broader number of patients.

Keywords: ATMP, tissue engineering, GMP, manufacturing, autologous, cartilage regeneration, automation & robotics, automation

INTRODUCTION

ATMPs are at the forefront of current state of the art medical science and technology. This innovative and complex class of biological products promises new therapeutic options for yet unmet medical needs. Currently, only 12 ATMPs hold European marketing authorization, mostly composed of Gene Therapy Medicinal Products (GTMP) and with merely two TEPs according to regulation EC1394/2007 (1, 2). Chronic and progressive diseases still pose major clinical challenges for conventional and even advanced therapies, especially for tissues with only limited regenerative capacity. Untreated injuries of articular cartilage for example may lead to progressive loss of cartilaginous as well as osseous tissue because of its limited ability for self-repair. Annually about two million people are affected by cartilage injuries in Europe and the United States alone, with significant effects on the patients' quality of life due to severe pain and impaired function, particularly in the joints (3). Moreover, if left untreated, these lesions predispose to the onset of osteoarthritis, which might ultimately necessitate total joint replacement. Even though results published for joint arthroplasty are generally satisfactory, 10–15% of the patients are dissatisfied and report complications (4). Furthermore, especially in younger patients (<60 years) the risk of revision surgery, associated with lower treatment efficacy, is increased by 20–35% in total (5, 6). Current treatments for focal cartilage defects, e.g., microfracturing or autologous articular chondrocyte implantation, are often associated with drawbacks such as limited defect sizes and donor site morbidity (7–9). Various new treatment approaches using ATMPs are currently under scientific investigation, also in clinical trials with mainly somatic cell therapy medicinal products (sCTMP) among others (10, 11).

MANUAL ATMP MANUFACTURING AND LIMITATIONS

A tissue engineering approach using autologous nasal septum derived cartilage cells, cultured on a 3D carrier matrix for treatment of focal cartilage defects reveals promising outcomes in a phase I (clinicaltrials.gov identifier: NCT01605201) and ongoing phase II clinical trial (clinicaltrials.gov identifier: NCT02673905) (12–14). These nasal chondrocyte tissue engineered cartilages (N-TECs) are combined ATMPs, consisting of autologous nasal chondrocytes, cultured on a certified, commercially available collagen membrane (Chondro-Gide®, Geistlich Biomaterials), and extracellular matrix produced by the cells.

The combined ATMPs are manufactured in a manual way, graphically depicted in **Figure 1A**, in a cleanroom facility according to GMP guidelines and Standard Operating Procedures (SOP). The demanding process relies on authorized manufacturing sites, highly trained personnel and rigorous quality controls in order to ensure continuous high product quality. In the first step a small cartilage biopsy is obtained from patient's nasal septum in an authorized clinical site. The biopsy is shipped to the manufacturing site by a validated transport procedure. Upon arrival, the cartilage sample is minced and chondrocytes are released from the biopsy by enzymatic digestion. The cells are expanded for 13 days in 2D *in-vitro* culture, which require daily medium changes for the first 3 days in the first expansion phase and medium changes every 3 days in the second expansion phase, including manual sampling for cell counting and microbial testing at the end of the expansion. Cells are passaged two times with four T175 cm^2 cell culture flasks required for each product, as described elsewhere (14). Once sufficient cell numbers are available, they are manually seeded at a defined density onto the scaffold to generate a 3D tissue in static culture. After a total culture period of 29 days, the TEP is tested and shipped to the clinical site and surgically implanted into the focal cartilage lesion (**Figure 1B**), to promote cartilage regeneration and mitigate disease onset (**Figure 1C**). All manual handling steps, such as biopsy mincing, media changes, cell seeding and sampling for in-process-controls and release tests, are currently conducted in an EU-GMP grade A (United States Food and Drug Administration (FDA)-Class 100) safety cabinet inside a grade B environment under laminar air flow. Despite highly qualified personnel, strict adherence to SOPs and an efficient process, current manual manufacturing is afflicted with several disadvantages regarding costs, scale up, reproducibility and standardization. The main cost drivers in the process are salary costs for qualified personnel during the labor-intensive process, as well as the operational costs for running a GMP cleanroom-facility. Although some cost reduction could be achieved by parallelization, there are clear limits to the upscaling of the process due to working hour restrictions and limited cleanroom capacity, additionally to an expectable shortage of qualified personnel. Another drawback in the manual manufacturing process is the limitation in terms of standardization and thus reproducibility. Critical steps like the distribution of cell suspension on the matrix surface requires extensive training, skills and experience to ensure homogeneity in cell distribution and equal matrix production throughout the scaffold. These particular steps are subject to inter- and intra-operator variability, additionally to the intrinsic variability of an autologous biological product and donor-batch-variations (15–17). This lack of standardization may affect the quality and reproducibility of the final product and also impedes the transferability of processes to other manufacturing centers. Moreover, the current open manufacturing system, the frequent manual process steps as well as the invasive final quality control testing, pose major risks for contaminations throughout the process chain. Overcoming these hurdles is a prerequisite to achieve a more time- and cost-effective, standardized and scalable automated manufacturing process.

AUTOMATED ATMP MANUFACTURING CONCEPT

Automation of ATMP manufacturing processes is a key technology to bring these translational pathways from bench to late phase III clinical trials and beyond. This perspective presents a concept for automation of the N-TEC process with the potential

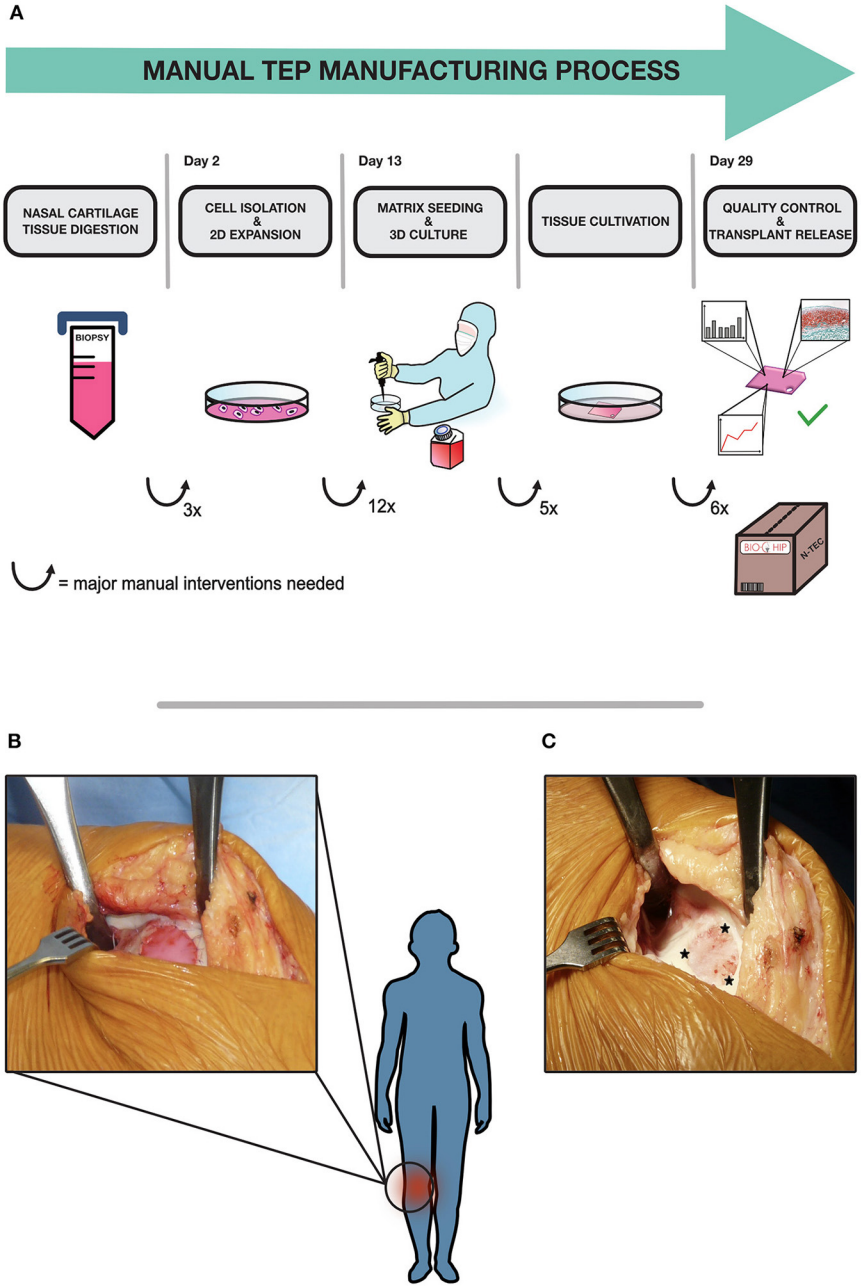

FIGURE 1 | BIO-CHIP manual manufacturing process and surgical procedure. The whole process is graphically depicted in **(A)**. An autologous cartilage biopsy is taken via outpatient surgery in a clinical site from the patients' nasal septum according to SOP. The biopsy is shipped to the manufacturing site, where the tissue is digested and the cartilage cells are isolated and expanded *in-vitro*. In the next step, cells are manually seeded on a collagen membrane in a certified cleanroom facility. Various parameters are monitored continuously throughout tissue cultivation. After 2 weeks of static tissue culture, final quality testing is conducted. The amount of extracellular matrix proteins is evaluated using histological grading by modified Bern score, also cell viability and transplant stability are assessed. When all defined release criteria are met, the N-TEC is packed and sent back to the clinic to be implanted into defect site in a surgical procedure **(B)**. The patch is secured by surrounding absorbable sutures during the surgical procedure. The initial focal cartilage defect in the knee is depicted in **(C)**, asterisks indicate the defect site where the N-TEC is inserted.

for a higher, more cost-efficient manufacturing capacity, process standardization and facilitated regulatory-compliant in-process documentation. The evolving field of ATMP manufacturing, especially during early process developments, necessitates a certain flexibility for such an automation concept. Although there are already many applications and disposable bioreactors available for suspension cell culture (18, 19), only very limited options exist for automated adherent cell culture (20). Moreover, even the production of sCTMPs like CAR-T or natural killer cells mainly relies on partly automated suspension culture systems

for clinical scale manufacturing (21–26). Additionally, the few commercially available semi-automated solutions for adherent cell culture systems require special single-use disposables additionally to high acquisition costs. Efforts on the part of the scientific community toward automated adherent cell production are currently investigated, with novel automated modular approaches for human mesenchymal stem cells by Kikuchi et al., or either commercially available products, like the description of the first adherent cell culture using CliniMACS Prodigy by Vieira et al. (27, 28). However, a fully automated platform that is highly adaptable to various specific steps, such as cell isolation involving mincing, or handling of cells from different donors in parallel without facing cross contamination issues, has not been described before. We propose a more adjustable design that is suitable for suspension cell culture as well as plate-based approaches, including adherent cell and tissue culture in a validated platform. The automated two arm robot design concept enables complete process automation of all manual process steps with standard laboratory equipment. Combined with necessary handling and storage units, quality control and regulatory framework in mind, the isolator is equipped with devices typically used in a tissue engineering facility, as shown in **Figure 2**. Our concept is also designed for automated cleaning procedures, a unique feature not addressed by other automated culture systems. To best of our knowledge, no fully automated manufacturing system is currently in use in academia nor other manufacturers of TEPs.

THE DESIGN OF AUTOMATION

The very center of the conception is based on a dual arm, six axis robot unit [**Figure 2A** (16), red]. Due to its high degree of freedom in movement, it allows for the implementation of complex tasks, and "human-like" robotic operations as liquid handling and cell culture (**Figures 2B,C**). Hence, manual protocols, previously developed in the lab, could be easily translated to be carried out by the robot as independent processes, without major alterations in equipment or process steps. The storage area (orange) is accommodated from an unclassified maintenance back side by linked glove box handling, without personnel entering the isolator directly. Pre-packed, sanitized disposables and materials can be unpacked easily and set in place for robot-driven procession. The concept follows a GMP-compliant unidirectional workflow, where all necessary disposables, liquids and starting materials (patient samples) enter the isolator through an air lock system and leave the aseptic environment through another air lock as final product. Patient samples are processed at a tissue culture area (green), where tissue mincing/digestion, cell isolation, seeding and tissue culture is conducted. Each handling step and consumable used in the process can be traceable through barcode-based recognition (09), ensuring continuous facilitated LOT-specific digital documentation and thus automating documentation. This automated continuous in-process documentation can significantly reduce mandatory regulatory paperwork, avoid sample mix ups and enhance product traceability as well as process transparency, eliminating time consuming manual protocol writing. On the direct opposite side, the sampling station (11) is located. This setup facilitates sampling during culture medium changes and further reduces handling distances of liquids within the operating plant. Samples are directly channeled through air locks to an adjoining quality control area (blue) [**Figure 2A**, (14,26)]. Trained members of the quality assurance unit accept the samples for manual inspection and testing. In this concept quality control of ATMPs is still conducted by experienced personnel, as the focus is on automating all steps involved in he N-TEC manufacturing. Ventilation systems (01) filter air through High Efficiency Particulate Air filter units (HEPA) and establish aseptic environmental conditions with only minimal amounts of airborne particle collectives. Vast incubator units (06) monitor and control humidity, air flow, CO_2-levels and temperature with storage space for cell culture plates for up to five TEPs at a time in parallel. Current manufacturing is heavily reliant on trained lab personnel whose interventions are also considered to be the main source of contamination in the aseptic manufacturing environment concerning FDA and European Medicines Agency (EMA). Implementing an isolator-type platform, allows for the separation of product and manufacturer, thus limiting human interaction and greatly reducing the risk of contamination. However, intricate cleansing by hand between production campaigns, clearly scotches these benefits nonetheless. Especially the performance of sequential manufacturing operations, with different patient material necessitates the development of quick and efficient cleaning procedures in between process steps and variant batch productions, to avoid cross contaminations according to EudraLex IV Point 4.26 (29). We propose to include automated cleaning procedures for all automated parallelized manufacturing systems of ATMPs. Current cleaning methods include gassing with hydrogen peroxide and a subsequent wipe-down or using other decontamination reagents during wipe-down. While gassing and wipe-down are thought to be possible to be implemented in an automated ATMP production plant, it is estimated that the whole procedure would take too long to be performed in between process steps handling cell material from different patients. To avoid cross contamination and accelerate the cleaning process, an approach based on spray nozzles is highly suggested. In similar approaches from the food industry, decontamination and cleaning reagents would be sprayed across surfaces and devices to remove any potential residual cell material. The platform is further equipped with a spray nozzle that may be used by the dual arm robot to clean any unsprayed areas [2A, (10)]. Afterwards the surfaces are dried with sterilized compressed air. Implementing such Cleaning-In-Place and Sterilization-In-Place based concept has great implications for the design of the production platform, as it has to be sealed off and proper drainage has to be achieved. As not all devices are suitable for such a procedure, the platform is compartmentalized into different modules according to their necessary functions. As of today, and to the best of our knowledge, such a GMP conform cleaning procedure has not yet been tested for automated ATMP manufacturing platforms and needs to be evaluated toward its efficacy and regulatory compliance.

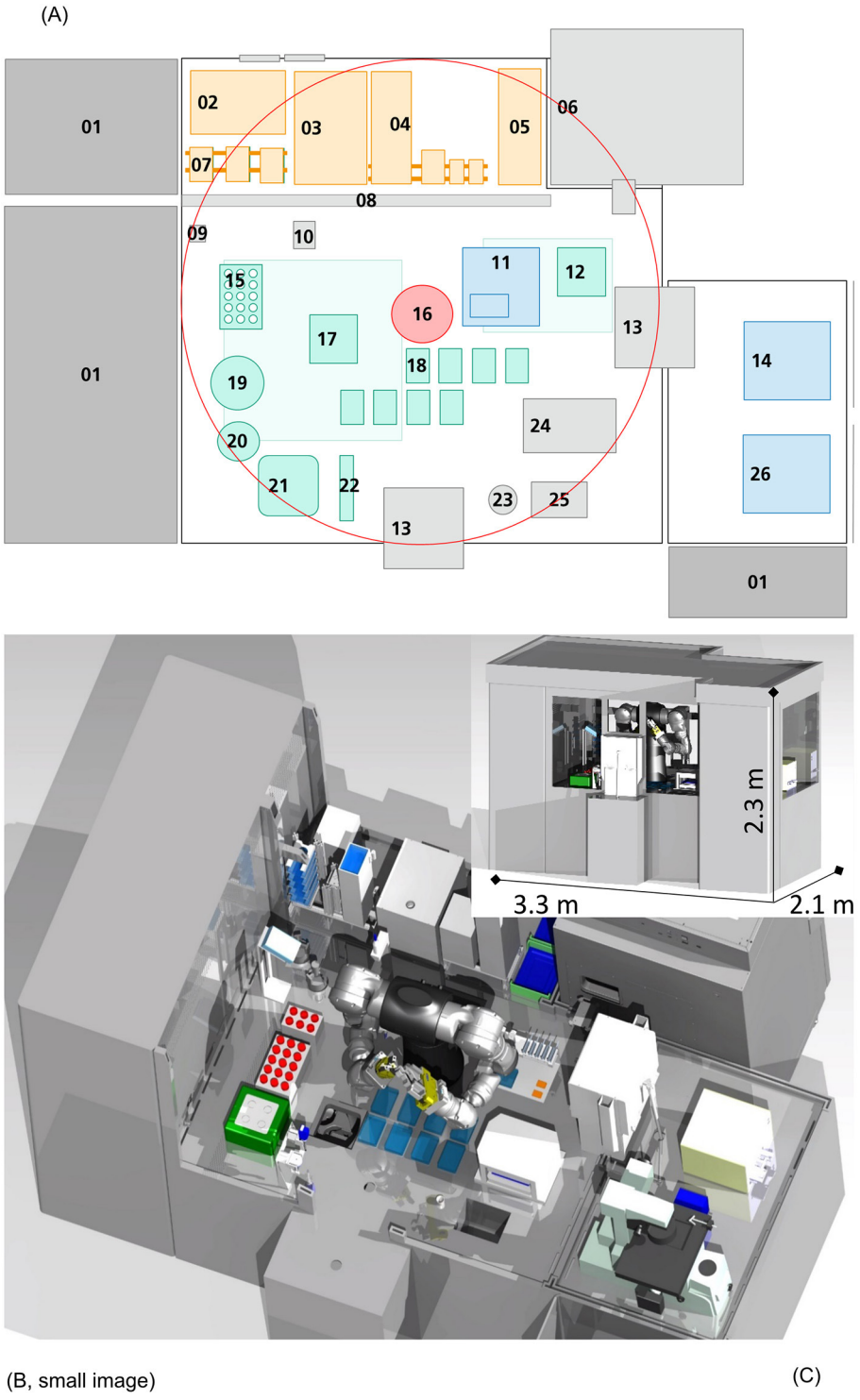

FIGURE 2 | Automation scheme and visual representation. In this figure, a 2D **(A)** and 3D [**(B)**: frontal view, **(C)**: sliced view] representation of this concept is shown. The platform is equipped with devices for each handling step: 01 Ventilation system, 02 Freezer (−20°C), 03 Fridge (4°C), 04 Disposables storage, 05 Packaging material, 06 Incubator (37°C), 07 Storage for plates and membranes, 08 Gate, 09 Barcode reader, 10 Washing station, 11 Sampling station, 12 Shaker, 13 Air-lock, 14 Cell counting device, 15 Storage for Cell culture tubes (temperature controlled), 16 Six axis dual arm robot, 17 Centrifuge, 18 Plate handling positions, 19 Decapper (centrifuge flask), 20 Decapper (Cell culture tubes), 21 Tissue grinder, 22 Pipettes, 23 Liquid waste, 24 Sealing machine, 25 Solid waste, 26 Microscope. The central six axis dual arm robot (16) can reach the circumference shown in **(A)**. Necessary equipment, disposables and liquids are safely channeled in through an air lock linked glove box (orange), without the need of personnel entering the isolator directly from an unclassified maintenance back side. Pre-packed, sanitized

(Continued)

FIGURE 2 | disposables, materials or biologicals can be unpacked easily and set in place for robot-driven procession. Devices for cell culture (green) and quality control (blue) are included in the design. The platform is scaled to the parallel production of five N-TECs at a time, with the possibility of increasing manufacturing capacity further e.g., by implementation of larger storage devices.

STANDARDIZATION FOR A MORE RELIABLE, AUTONOMOUS PRODUCTION

Another challenge of the current manual production is the potential inter- and intra-operator variability during seeding of the scaffold and to a lesser extent the cartilage digestion. Thus, automation of these steps has great potential, in particular to reduce variations due to operator handling in the final product. In the proposed automation concept, cell seeding is performed by the dual arm six axis robot. However, the complexity of the cell seeding process will require careful implementation and meticulous testing before standardization by automation of this step is achieved. In the long term, standardization of this process step would reduce the process-induced variability and allow for a more reproducible and high-quality production. In general, the most complex steps are the ones most relevant for automation. But also automation of time consuming repetitive steps, like manual medium exchange, could quickly impact on cost-effectiveness. Moreover, the risk of human error is reduced to an absolute minimum when handling production in a closed automated system in contrast to open manual handling.

AUTOMATION AND COST-EFFECTIVENESS

Especially with constantly rising personnel wages, an automated production will become even more cost-effective from an economic perspective. In the complex production of iPS cells, personnel costs account for nearly 60% of total manufacturing costs, with estimably 42% more manual production costs than in automated production (30). These costs are comparable to a sophisticated TEP product like the N-TEC, which ranges from 17,000–20,000 € per product with frequent manual interventions and produced in an academic setting. The investment for automated iPS production is estimated to be about 1,000,000 €, whereas the investment for the automated TEP production plant in total is estimated 1,500,000 €, with additional operational resources as described elsewhere (30). Even though the initial payback period a TEP-facility might also be longer, than those of a cell production line, with an increasing TEP market availability, positive cash flow could be achievable within the first 3 years of market acceptance. Parallelization could further lift single-cost burdens, and greatly benefit to a fast scale up process. Additionally, an automated production platform is independent of working hour restrictions and can be designed to a very confined space, superseding cost-intensive walk-in cleanroom structures.

ATMP MANUFACTURING 4.0: FACILITIES OF THE FUTURE

The main objective of this work is the mere automation of a former manual process with a rather deterministic robotic unit. This transitional step could pave the way for future technology and applications that will further improve the production process. Our current ATMP manufacturing protocol and the concept for the automated platform incorporates invasive sampling procedures prior to product release and manual scoring to ensure high product standards to pre-defined quality criteria. Although not yet integrated in the concept, first approaches to automate these controls have already been carried out. As an example, the automated visual inspection of histological tissue engineered cartilage using a modified Bern score and deep learning algorithm has already been demonstrated to be a feasible method for the prospective evaluation and graft release in a clinical manufacturing setting (31, 32). However, these pivotal procedures would greatly benefit from the implementation of non-invasive in-process controls, enabling real-time quality control and monitoring of product specifications throughout the manufacturing process. Appropriate methods using non-invasive sampling e.g., supernatant for cell viability determination through lactate dehydrogenase assay, are currently reviewed and validated for GMP compliant manual application. Other possible technologies for such a non-invasive quality control would include optical coherence tomography (OCT) or Raman spectroscopy, which are currently under evaluation regarding quality controls for TEPs prior to implementation in the process (33). The ultimate goal is to monitor and collect high quality product related data (e.g., cell population doubling, temperature, oxygen, pH) through equipped sensors in order to employ data driven approaches for process and schedule optimization. Non-invasive continuous monitoring along with model-based strategies have the potential to supersede current invasive quality monitoring and to enable a true "smart factory" setting. *In-silico* tools employing artificial intelligence to predict process outcome might be used to predict the best harvesting time points, before cells have reached critical mass (34). This would be highly beneficial for the quality and outcome of *in-vitro* generated tissues by accounting for the individual needs and nature of patient derived cells, but also facilitate the critical release testing. With product data sets from continuous monitoring, *in-silico-predictions* could finally allow a near real-time release of TEPs matching individually scheduled dates for surgical procedures, without the risk of delay due to product inconsistencies.

DISCUSSION

Many ATMPs are currently under review and in preparation for at least partly automated manufacturing systems, on the market as well as in research and clinical facilities. But we also observe from experience, that the latter is introduced far beyond the point of product development, in late stages of testing which makes it difficult to develop a platform that is adaptable to all needs of the product manufacturing. We propose to keep the concept of automation always in mind when initially developing a manufacturing process to ease initial implementation, and

to avoid unnecessary expenses for product revalidation (35). Dual-use equipment for example, which is suitable for manual laboratory work, but also eligible to be implemented in an automated setting, would prevent doubling the high acquisition costs for a manual and automated process later on. The question how far a biological process can really be improved above the limits of inherent biological variance is a tough one. It can only be addressed properly, when such an automated manufacturing system has actually been built and successfully been tested in full extent. Currently, automation concepts are hindered by comparatively small technical pitfalls, such as the lack of automated cleaning procedures. However, they also add on the whole new topic of validating of the automation software and procedures according to good automated manufacturing practice (GAMP). Several automated platforms and devices have been developed for cell culture and ATMP production, however these are either reliant on disposable inserts or hazardous manual cleaning procedures that necessitate an interruption between production campaigns. Automated manufacturing becomes more cost-effective especially when parallelizing production to a high extent. To avoid long down-times before, and cross contamination during production, automated cleaning procedures must be developed and validated for these platforms. Moreover, technical challenges in facility design for these cleaning procedures must be overcome, wherefore more research and development in that area is needed. But is it worth the effort in the end? Early cost-intensive investments in automation may seem irrational at first, but will pay off in form of a consistent process for phase I, and more scalable product for phase II/III clinical trial stages. We conclude that implementing 4.0 standards and new manufacturing methods to TEP and ATMP production in general must be more than just a scientific exercise. Automation will help to unleash the full economic potential of ATMPs in an ever competitive drug market. Only then, with a positive cost-benefit-ratio, living drugs will be appealing to health care providers and insurances. This will ultimately help to deliver more ATMP based therapies to patients in dire need.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SH wrote the first draft of the manuscript. LH and SH created the figures. SH, LH, SM, and AW wrote sections of the manuscript. All authors contributed to the conception and design of the perspective, contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This project received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 681103, BIO-CHIP. This publication was supported by the Open Access Publication Fund of the University of Wuerzburg.

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frontiers in Medicine

REVIEW
published: 16 August 2021
doi: 10.3389/fmed.2021.713401

Production and Application of CAR T Cells: Current and Future Role of Europe

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Rapid developments in the field of CAR T cells offer important new opportunities while at the same time increasing numbers of patients pose major challenges. This review is summarizing on the one hand the state of the art in CAR T cell trials with a unique perspective on the role that Europe is playing. On the other hand, an overview of reproducible processing techniques is presented, from manual or semi-automated up to fully automated manufacturing of clinical-grade CAR T cells. Besides regulatory requirements, an outlook is given in the direction of digitally controlled automated manufacturing in order to lower cost and complexity and to address CAR T cell products for a greater number of patients and a variety of malignant diseases.

Keywords: CAR T cells, cancer treatment, manufacturing, automation, regulation

OPEN ACCESS

Edited by:
Hans-Dieter Volk, Charité Medical University of Berlin, Germany

Reviewed by:
Dimitrios Laurin Wagner, Charité – Universitätsmedizin Berlin, Germany
Yu Qian, University of Texas MD Anderson Cancer Center, United States

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *22 May 2021*
Accepted: *23 July 2021*
Published: *16 August 2021*

Citation:
Vucinic V, Quaiser A, Lückemeier P, Fricke S, Platzbecker U and Koehl U (2021) Production and Application of CAR T Cells: Current and Future Role of Europe. Front. Med. 8:713401. doi: 10.3389/fmed.2021.713401

CELL-BASED CANCER THERAPY: FROM STEM CELL TRANSPLANTATION TO PERSONALIZED THERAPY WITH CAR T CELLS

The basis for cell-based cancer therapies was laid with the development of allogeneic hematopoietic stem cell transplantation (HSCT) in the 1960s (1)(1). From those beginnings to the present day, more than a million HSCTs have been performed around the world (2). After intensive conditioning therapy (chemotherapy or radiation), donor hematopoiesis is established as well as a graft-vs-tumor effect (3), as a result of which the donor's T lymphocytes recognize cancer cells as foreign and can kill them by various mechanisms. This effect was also described following the administration of donor lymphocyte infusions (DLI) for relapse treatment (4). However, differences in the HLA and/or minor histocompatibility antigens between donor and recipient can also trigger graft-vs-host disease (GvHD), which represents one of the most serious complications after allogeneic HSCT and can affect almost every organ system (5). In addition to identifying HLA-identical family donors, large registers are used to specifically search for HLA-compatible third-party donors. For many patients without HLA-compatible donors, haploidentical transplantation (donor and recipient share half of the HLA characteristics) is an established alternative. Despite a large number of foreign HLA antigens, T cell depleting drugs such as cyclophosphamide, applied immediately after transplantation, can reduce acute or chronic GvHD, with survival rates comparable to conventional HSCT (6, 7). The function and activity of the T cells in a hematopoietic cell transplant and the immune cells that develop after engraftment

of the stem cells are therefore essential components of therapeutic success: an increase of the anti-tumor efficiency with simultaneous elimination or significant reduction of the T cell-mediated side effects (e.g., GvHD, cytokine release syndromes) is optimal. This has led to the development of the principle of modulating T cells as an essential part of immuno-oncological research and the generation of new therapeutic agents.

New antibody therapies are also making use of the impressive clinical potential of T lymphocytes. Checkpoint inhibitors such as ipilimumab, nivolumab, or pembrolizumab are monoclonal antibodies, the binding of which leads to the abolition of a mostly tumor-induced inhibition of T lymphocytes and thus to a therapy response (8). Furthermore, bispecific antibodies, which bind T cells in addition to the target antigen, are considered to be further developments of this principle (9). One example is blinatumomab, which is approved in the treatment of refractory or relapsed precursor B-cell lymphoblastic leukemia (r / r B-ALL) (10). This dual antibody fragment has binding sites both for CD19 (another antigen on B-ALL and B-lymphoma cells) and against CD3 (part of the T cell receptor) and thus leads to the formation of an immunological synapse between cancer cells and cytotoxic T lymphocytes (11).

In analogy to bispecific antibodies, certainly more complex reprogramming of T-lymphocytes can also be performed through transfer of the genetic information of an antibody-binding domain fused to essential T cell signaling domains, in context of therapy with CAR (chimeric antigen receptor) T cells. In this process, autologous T lymphocytes of the patient which recognize the target antigen are produced *ex vivo* through viral transduction of the CAR-T cells (12). These so-called living drugs were approved in USA 2017 and in EU 2018 are one of the most innovative therapy options for the treatment of aggressive B-cell lymphomas and the precursor B-ALL (<25 years).

After transduction, these cells express a variable domain of immunoglobulin, which, as an antigen receptor, is specifically directed against the surface antigens of cancer cells (13). Since these immunoglobulins are not physiologically expressed on T cells, these genetically modified T cells are also referred to as CAR T cells. Another difference to the natural T cell receptor is the fusion of costimulatory domains to the CAR molecule, which increase the efficacy of the cells (**Figure 1**) (17, 18).

Theoretically, it is possible to generate CAR T cells against a large number of relevant tumor antigens, nicely reviewed in (19–22). Once the tumor antigen has been recognized, the CAR T cells are activated, resulting in a targeted immune reaction directed against the respective tumor.

CLINICAL APPLICATION OF CD19-CAR T CELLS

One of the first clinical applications of CD19-CAR in hematology took place in 2009 in an intensively pretreated patient with follicular lymphoma (FL) where a partial remission was achieved by using CAR T cells (23). In 2010, the University of Pennsylvania started the first phase I study for adult patients with mature B-cell neoplasms (24). After the inclusion of three patients with chronic lymphatic leukemia (CLL), the study was stopped for financial reasons. An additional problem was the management of inflammatory reactions, summarized under the term cytokine release syndrome (CRS) (25). This may lead to life-threatening complications such as insufficient oxygen supply with the need for ventilation, severe hypotension with reduced blood flow to the periphery requiring circulatory support therapy, capillary leak syndrome with edema formation, especially of the lungs, but also multi-organ failure and disseminated intravascular coagulopathy (26). The publication of the positive clinical results of these patients (2 complete and 1 partial remission) lead to an increased global interest in CAR T cells (27). The goal of developing personalized immunotherapies and translating them into clinical application led to a cooperation between the University of Pennsylvania and Novartis in 2012, followed by a partnership between Kite Pharma and the National Cancer Institute (NCI) (28, 29). In 2013, the treatment results of two first pediatric patients with refractory or relapsed acute lymphoblastic leukemia (r/r ALL) were published (30). In addition, this was the first publication on the successful application of tocilizumab (anti-IL-6 antibody) in severe CRS. Further studies confirmed the surprisingly good complete remission rates in this patient cohort, which was previously considered as treatment refractory and thus incurable. (31). Additionally, the possibility of achieving a permanent remission for r/r ALL patients could be proven in a global multicenter study (25 centers in 11 countries) (32). The efficacy of CAR T cells has also been demonstrated in patients with lymphomas. The first phase II study was started at the University of Pennsylvania in 2014 in patients with r/r DLBCL and FL (33), followed by two multicenter international phase II studies for patients with refractory or recurrent diffuse large-cell B-cell lymphoma (r/r DLBCL) (34, 35). However, CAR T cell therapy may be associated with other complications in addition to CRS, such as immune effector cell-associated neurotoxicity syndrome (ICANS) and the macrophage activation syndrome. According to current recommendations from specific specialist societies, to treat CRS and to prevent this complication from progressing further, anti-IL-6 antibodies are given in its early stages (25, 36). For treatment of ICANS without CRS, corticosteroids are the therapy of choice. The standardized, stage-appropriate therapy of these possible complications requires the full-day availability of the anti-IL-6 antibodies in the clinic, as well as an interdisciplinary team for the immediate initiation of intensive medical, neurological and imaging measures, but also the continuous training of nursing and medical staff as summarized in the EBMT/ISCT recommendations (37, 38).

Three preparations are currently approved in the EU: tisagenlecleucel (Kymriah®) (39) and axicabetagene ciloleucel (Yescarta®) (40) for treatment of pediatric patients with r/r primary mediastinal B-cell lymphoma following at least two previous lines of therapy and brexucabtagene autoleucel (Tecartus®) (41) for treatment of mantle cell lymphoma in adult patients. EU approval for further drugs with other target antigens, e.g., B-cell maturation antigen, is expected in 2021.

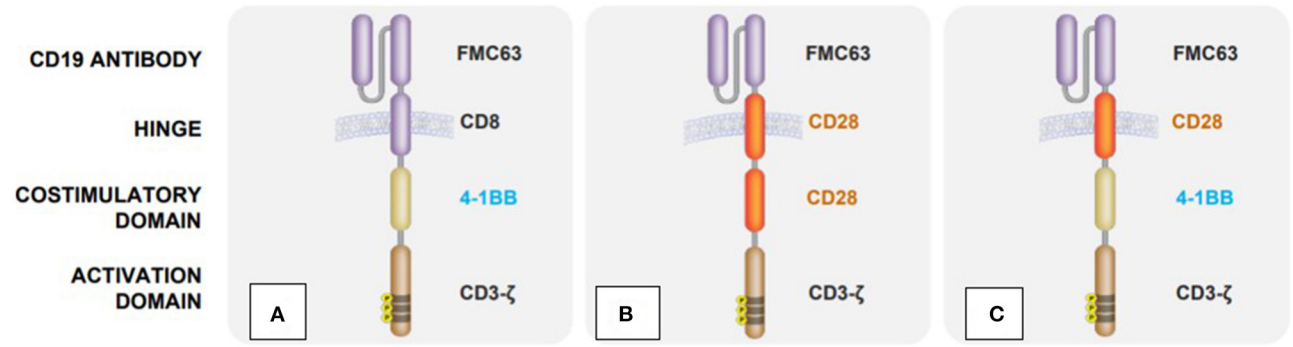

FIGURE 1 | Structural differences between clinically available CAR T cell products: **(A)** Tisagenlecleucel (Kymriah) (14), **(B)** Axicabtagene ciloleucel (Yescarta) (15), **(C)** Lisocabtagene maraleucel (Breyanzi) (16).

So far, various pediatric and internal medicine centers have been certified for treatment with CAR T cell therapies that are associated with considerable additional logistical and infrastructural efforts. The number of centers varies in the individual EU countries depending on the organization of the health care system. There are only a few centers in centrally organized systems, whereas Germany with its decentralized, area-wide medical care concept has 26 centers (42). In Germany alone, CAR T cells for the treatment of patients with r/r CD19^{+} ALL/DLBCL are needed for approx. 1,200–1,400 patients per year (43).

CAR T CELLS AS CLINICAL TRIAL PRODUCTS: RULES, CONDITIONS, AND GLOBAL DEVELOPMENT

From a regulatory point of view, CAR T cells are an advanced therapy medicinal product (ATMP) in the EU. ATMPs are classified in (i) gene therapy medicinal products, including CAR T cells (ii) somatic cell therapy medicinal products, (iii) tissue-engineered products and (iv) combined ATMPs. They play a growing role in the treatment of cancer and hereditary diseases as well as in regenerative medicine and, more recently, in the development of therapies for viral infections. CAR T cells as an ATMP can be generated by either viral transduction leading to a permanent CAR expression or by using mRNA as well as transposon technology for transient CAR expression.

The manufacture, approval, and regulation of these innovative therapies are extremely complex and serve to protect the patients. They are subject to health and research policy framework as well as legal regulations that have a direct influence on international competitiveness. Therefore, the design of the framework is an important instrument to support research in the EU and to promote innovations. This, however, needs to be considered also in the context of international activities. The European Parliament and the Council of the European Union have issued Regulation (EG) No. 1394/2007 that regulates licensing, monitoring, and pharmacovigilance of ATMPs (44). Central approval is compulsory in the countries of the EU offering the advantage of market access in all EU member states. However, different regulatory frameworks within individual member states lead to complexity and reduce competitiveness. In Germany, for example, there are stricter regulations for the import of medicinal products and active ingredients from third countries than required by EU regulations (AMG § 72a). As a result of the lack of international harmonization in the recognition of certificates, manufacturers are obliged to carry out an acceptance inspection of the apheresis unit in non-EU countries. On the one hand, this obstacle affects the supply of CAR T cells for patients and, on the other hand, orders from abroad are lost, even if the manufacturer has a high level of professional qualification. The federal system that exists in Germany is also not conducive at this point. For example, the granting of a manufacturing license (AMG § 13) is subject to the respective state authority of the federal state and must be applied for a new in each other. Carrying out academically initiated studies requires considerable financial and human resources which University hospitals are currently unable to cover for the most part. Funding programs for such high financial volumes are only available to a limited extent. Due to the special nature of the production, there is an increased dependence on the industry. This situation prevents academic studies in Europe.

Currently, there are around nine hundred studies worldwide with the use of CAR T cells as investigational drugs in different tumor entities, which confirms the increasing interest in immuno-oncology (45). There is good reason to hope that, in addition to addressing CD19+ hematological diseases, the development of therapies in oncology will also increase rapidly. The current Biotech Report of the Boston Consulting Group shows that only around 10% of studies are coordinated in Europe (46). Currently, China is the leader in this field, followed by the USA. Europe has already fallen behind its competitors and substantial investments & regulatory reforms are required to catch up. A look at the financing reveals a serious difference. While in Europe an average of 60 percent of the studies are

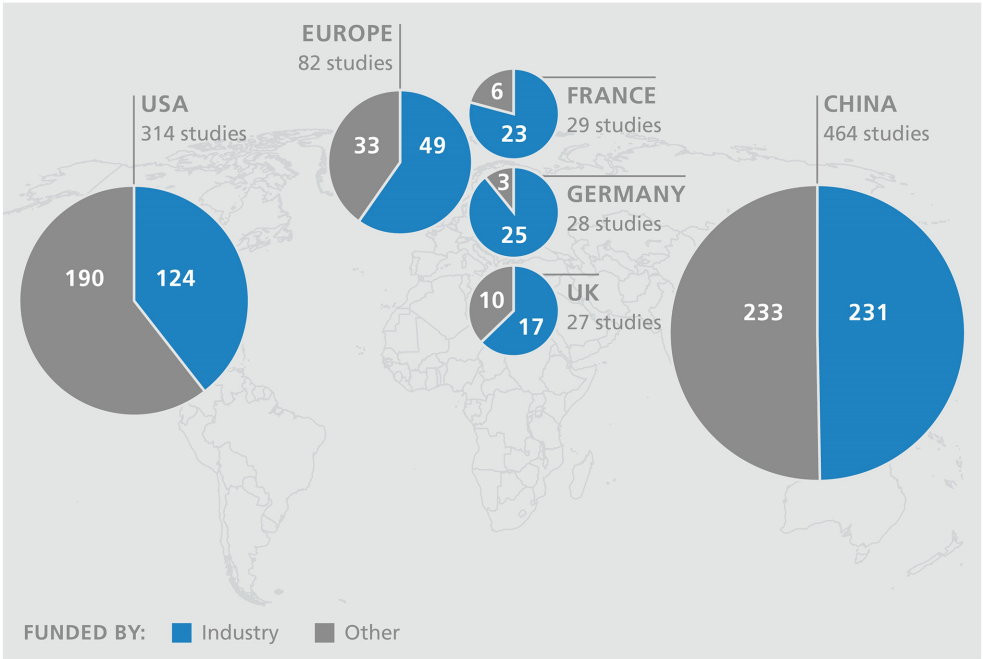

FIGURE 2 | Top 5 countries of clinical CAR T cell studies with funding type (source: ClinicalTrials.gov).

sponsored by industry, the level is even significantly higher in some member countries (Germany 90%), in the USA and China more than half are initiated from the academic sector (**Figure 2**). In addition, a large amount of venture capital or governmental funding are available for the subsequent implementation in the USA and China - there are hardly any comparable options in Europe.

Furthermore, reliable reimbursement conditions and price setting must be put on the political agenda so that (i) patients can be guaranteed access to standard care with these high-priced therapies in the future (∼1/4 million EUR/product for both approved CAR T cell products) without overwhelming the solidarity community and (ii) the financing gap in the clinics can be closed.

An innovative financing model was proposed by the pharmaceutical industry, which as so-called "Pay-for-Performance" provides for payments only if the treatment is successful (47). Manufacturing in strong networks using both centralized and decentralized manufacturing gives rise for future financial opportunities (48).

Government programs and financial support make a substantial contribution to support independent research and help to implement innovative ideas. In the field of cell and gene therapy, this could not only have a major influence on price formation of ATMPs, but would also strengthen the European position in this area. In its research and innovation investment program "Horizon Europe" approved at the beginning of the year, the European Commission provided a total budget of 95.5 billion euros, of this 25 billion earmarked for promoting scientific excellence.

TECHNOLOGY DEVELOPMENT AND TRANSFER

So far, the focus has largely been on the development and improvement of the product, so the innovations are aimed at (i) CAR construct design up to the fourth generation, the TRUCK (T cells directed for antigen-unrestricted cytokine-initiated killing), (ii) non-viral vector formats like the Sleeping Beauty (SB) Transposon System and (iii) switchable universal CAR T platform technology (UniCAR), which allow to repeatedly turn the activity of CAR T cells on and off (49–51). However, the manufacturing process with its complex sequence of different process steps (1.) cell preparation, such as thawing and washing, (2.) selection, (3.) activation, (4.) transduction, (5.) expansion, (6.) harvest and (7.) final formulation of the cells, is still in an early development phase. Most of the products are manufactured under manual and only partially automated conditions.

After market approval of the first two CAR T cell preparations, the existing infrastructure is simply used for their production, which takes place in cooperation between the large pharmaceutical and biotechnology companies and their partners. In a second step, highly qualified specialist institutions are commissioned to guarantee the supply. In Germany, for example, there has been a collaboration between Novartis and the Fraunhofer Institute for Cell Therapy and Immunology (IZI) for the production of CAR T cells, initially as clinical test preparations, since 2015. So far, more than 500 CAR T cell preparations have been produced at IZI as part of this cooperation. There are currently about 150 qualified treatment centers in 20 countries in Europe including the

TABLE 1 | Automated CAR T cell production: Publications and clinical trials (14, 53, 54, 56–73).

Author (year of publication)	Title	Device platform	Product/ Runs
a) Reviews			
Fritsche E. et al. (73)	Toward an Optimized Process for Clinical Manufacturing of CAR-Treg Cell Therapy	1. GMP compliant cell sorter 2. Bioreactor 3. CliniMACS Prodigy®	
Mizukami A. and Swiech K. (72)	Platforms for Clinical-Grade CAR-T Cell Expansion Book: Chimeric Antigen Receptor T Cells (Chapter 10)	1. Bioreactor 2. CliniMACS Prodigy® 3. Octane Cocoon^{TM} cell culture system	
Smith D. et al. (70)	Toward Automated Manufacturing for Cell Therapies	1. Bioreactor 2. CliniMACS Prodigy® 3. Octane Cocoon^{TM} cell culture system	
Smith TA. (71)	CAR-T Cell Expansion in a Xuri Cell Expansion System W25 Book: Chimeric Antigen Receptor T Cells	Xuri Cell Expansion System W25	
Roddie C. et al. (69)	Manufacturing chimeric antigen receptor T cells: issues and challenges	1. Wave Bioreactor 2. G-Rex flask 3. CliniMACS Prodigy®	
Moutsatsou P. et al. (55)	Automation in cell and gene therapy manufacturing: from past to future	1. CliniMACS Prodigy® 2. Octane Cocoon^{TM} cell culture system 3. Quantum Cell Expansion (hollow fibers)	
Iyer R.K. et al. (74)	Industrializing Autologous Adoptive Immunotherapies: Manufacturing Advances and challenges	1. G-Rex static bioreactor 2. Wave-mixed Bioreactors 3. CliniMACS Prodigy® 4. Octane Cocoon^{TM} cell culture system 5. Quantum Cell Expansion (hollow fibers)	
Piscopo N.J. et al. (68)	Bioengineering Solutions for Manufacturing Challenges in CAR T Cells	1. Bioreactors 2. CliniMACS Prodigy®	
Kaiser A. et al. (67)	Toward a commercial process for the manufacture of genetically modified T cells for therapy	CliniMACS Prodigy®	
b) Paper			
Costariol E. et al. (66)	Demonstrating the Manufacture of Human CAR-T Cells in an Automated Stirred-Tank Bioreactor	Stirred tank bioreactor	CD19 CAR-T Donors (n =3)
Jackson Z. et al. (56)	Automated Manufacture of Autologous CD19 CAR-T Cells for Treatment of Non-Hodgkin Lymphoma	CliniMACS Prodigy®	CD19 CAR-T trial participants (n = 31)
Castella M. et al. (65)	Point-Of-Care CAR T-Cell Production (ARI-0001) Using a Closed Semi-automatic Bioreactor: Experience From an Academic Phase I Clinical Trial	CliniMACS Prodigy®	CD19 CAR-T trial participants (n = 28)
Fernández L. et al. (64)	GMP-Compliant Manufacturing of NKG2D CAR Memory T Cells Using CliniMACS Prodigy	CliniMACS Prodigy®	NKG2D CAR Memory T Cells validation runs (n = 4)
Vedvyas Y. et al. (63) Erratum in (2020)	Manufacturing and preclinical validation of CAR T cells targeting ICAM-1 for advanced thyroid cancer therapy	CliniMACS Prodigy®	ICAM-1 CAR-T preclinical validation (n = 7)
Aleksandrova K. et al. (53)	Functionality and Cell Senescence of CD4/CD8-Selected CD20 CAR T Cells Manufactured Using the Automated CliniMACS Prodigy® Platform	CliniMACS Prodigy®	CD20 CAR-T establishing runs (n = 6)
Zhang W. et al. (62)	Characterization of clinical grade CD19 chimeric antigen receptor T cells produced using automated CliniMACS Prodigy system	CliniMACS Prodigy®	CD19 CAR-T establishing run (n = 1)
Blaeschke F. et al. (52)	Induction of a central memory and stem cell memory phenotype in functionally active CD4(+) and CD8(+) CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19(+) acute lymphoblastic leukemia	CliniMACS Prodigy®	CD19 CAR-T autologous patients (n = 4)
Zhu F. et al. (61)	Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS Prodigy device at an academic Medical Center	CliniMACS Prodigy®	CD19 und CD20/CD19 CAR-T test runs (n = 7)
Lock D. et al. (60)	Automated Manufacturing of Potent CD20-Directed Chimeric Antigen Receptor T Cells for Clinical Use	CliniMACS Prodigy®	CD20 CAR-T test runs (n = 15)

(Continued)

TABLE 1 | Continued

Author (year of publication)	Title	Device platform	Product/ Runs
Priesner C. et al. (59)	Automated Enrichment, Transduction, and Expansion of Clinical-Scale CD62L(+) T Cells for Manufacturing of Gene Therapy Medicinal Products	CliniMACS Prodigy®	GFP- T proof of principle $n = 3$ (4)
Mock U. et al. (58)	Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy	CliniMACS Prodigy®	CD19 CAR-T test runs ($n = 7$)
c) Clinical Trials			
NCT04196413	GD2.BB.z.iCasp9-CAR T Cells	CliniMACS Prodigy®	$n = 54$
NCT03467256	CD19 CAR-T	CliniMACS Prodigy®	$n = 18$
NCT04049383	CAR-20/19-T cells	CliniMACS Prodigy®	$n = 24$
NCT03144583	CD19 CAR-T	CliniMACS Prodigy®	$n = 28$
NCT03434769	CD19 CAR-T	CliniMACS Prodigy®	$n = 31$
NCT03893019	CD20 CAR-T	CliniMACS Prodigy®	$n = 15$
Unknown	CD19 CAR-T	Cocoon® Platform	$n = 1$

UK, plus more than 200 outside the EU (52). The global supply of tisagenlecleucel (Kymriah®) is provided by seven manufacturers, 3 from Europe (France, Switzerland, Germany) and the others from the USA, China, Japan and Australia. In parallel, the same number of centers are qualified for the treatment with Yescarta®. To cope with the forecast, increase in the number of treatments, there is an urgent need for automation. Given the possibility to address not only hematological diseases, but also solid tumors, as a result, around 1.5 decimal powers more CAR T cell preparations must be made available. Upscaling is not a trivial process, but requires the optimization of each individual step and the analysis of the effects on the product by corresponding complex in-process and final product controls. In publications of some research groups, influences on the phenotype, exhaustion and senescence of the cells are described, which can lead to functional limitations (53, 54). Understanding molecular mechanisms is an important component in the development of new process strategies. An evaluation of clinical studies from the past 15 years reflects the diversity within the production of CAR T cells (55). This variability should be minimized to achieve a uniform robust process. In initial optimizations, open steps were replaced by closed steps in order to decimate the risk of product contamination. By reducing manual steps, which are extremely time-consuming and require the use of well-trained and highly qualified staff, the aim is now to increase the efficiency of implementation. The CliniMACS Prodigy® from Miltenyi Biotec, for example, offers the possibility of decentralized production and is already being used as a proof of concept in several ongoing clinical trials for the production of CAR T cells (**Table 1**). This automatic and closed device is able to map all process steps from cell preparation to harvest.

However, the small chamber volume, the insufficient flexibility and the restricted use, which occurs during the cell expansion phase of several days, have a limiting effect and could lead to a production bottleneck (56). In efforts to shorten the process, the cultivation time has been reduced from the usual 12 days to 8 days (57). Another automated system, the Cocoon® platform from Lonza, was first used successfully last year at the Sheba Medical Center in Israel within a clinical trial (75). Alternatively, modular systems are used. Devices from various manufacturers, which only perform the respective process step automatically, are combined as needed. Widely used, even in commercial production, is the use of bioreactors for cell expansion. They can become a key element of industrialized manufacturing, as the new generation allows control of culture conditions and the possibility of process adaptation (74). The involvement of continuous monitoring of relevant process parameters and defined cell patterns would enable an adaptive process management. As an example, the Prodigy device is equipped with a microscope camera that already allows continuous monitoring of cell growth within the chamber. For the future, automated daily harvesting of small samples, which are transferred with a robotic arm into an external machine for cell characterization could improve early decision during a manufacturing process. Thus, the influence of subjective decisions and human-related protocol deviations could be minimized or eliminated (58). A modular system offers the decisive advantage of being able to organize the processes flexibly. The platforms are still not networked with one another in order to automatically map the entire process chain. The integration of different device and technology platforms for production and quality control in a digitally controlled process line would offer the flexibility and automation required for a large number of diverse cell and gene therapeutics and adaptations to further developments (56).

On the question of whether to favor centralized or decentralized manufacturing, existence of both is justified. In development and translation to the clinic, decentralized manufacturing in qualified GMP facilities of University hospitals plays an important role. The challenge in the commercialized manufacture of personalized therapies lies in the creation of various parallel independently running product manufacturing processes. This complexity calls for centralizing commercial production. Experience from other industrial sectors and the

potential of the industry 4.0, characterized by the digitalization of production, can help to break new ground in the direction of robotic systems and intelligent automated process lines. Investments in the development of strategies for the automation and digitization of the production, product control and documentation of ATMPs must play a central role so that the global supply of patients with cell and gene therapeutics can be guaranteed in terms of availability of capacities, resources and finances.

CONCLUSION

With its excellent and diverse research landscape, Europe still plays an important role worldwide. In contrast, more than 90 percent of clinical trials with CAR T cells are currently initiated outside Europe. Compared to the U.S. and China, venture capital funding is underdeveloped in Europe and regulations, decision processes and initiation of studies are lengthy and complex. The creation of appropriate framework conditions in an international context therefore seems essential to address and overcome (i) the delayed translation of research into the clinic, (ii) the lack of funding but also the increasing complexity of academically initiated phase I/II clinical trials, and (iii) improved support in the developments of automation and digitization of process routes in order to address 100-fold more patients moving from haematological to solid cancer. In the end, this will also determine how strongly Europe will be represented in the economic value added in the promising market of cell and gene therapy. Policymakers are therefore faced with the question of the extent to which they support science and create the conditions that are conducive to innovative developments in order to ultimately strengthen Europe as a location for research and business and not lose touch with the world leaders. Funding programs, such as Horizon Europe pave the way for better networking and cooperation among member states. However, efforts toward international harmonization of regulations must also be accelerated, because ultimately the huge challenges in the development and provide of personalized medicines cannot be met by national efforts alone, but only within the framework of international cooperation.

AUTHOR CONTRIBUTIONS

VV and AQ wrote the manuscript. PL, SF, UP, and UK provided administrational support. All authors listed approved the manuscript for the publication.

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Conflict of Interest: AQ and PL declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. VV discloses honoraria for Novartis, Gilead, BMS, and travel grants from Gilead. SF states honorary activities for Novartis. With regard to the production of cell therapeutics, there are cooperations with the companies Novartis and Miltenyi Biotec. UP discloses consulting and fee for service activities for Novartis, Gilead and BMS. UK states that she is a consultant in immuno-oncology for AstraZeneca, Affimed, Glycostem, GammaDelta and Zelluna, and that she has collaborations with Novartis and Miltenyi Biotec regarding the production of CAR-T cells.

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frontiers in Medicine

HYPOTHESIS AND THEORY
published: 13 September 2021
doi: 10.3389/fmed.2021.728866

An Individual Patient's "Body" on Chips—How Organismoid Theory Can Translate Into Your Personal Precision Therapy Approach

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Reviewed by:
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Institute of Biomedical Chemistry, Russia
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: 22 June 2021
Accepted: 16 August 2021
Published: 13 September 2021

Citation:
Marx U, Accastelli E, David R, Erfurth H, Koenig L, Lauster R, Ramme AP, Reinke P, Volk H-D, Winter A and Dehne E-M (2021) An Individual Patient's "Body" on Chips—How Organismoid Theory Can Translate Into Your Personal Precision Therapy Approach. Front. Med. 8:728866. doi: 10.3389/fmed.2021.728866

The first concepts for reproducing human systemic organismal biology *in vitro* were developed over 12 years ago. Such concepts, then called human- or body-on-a-chip, claimed that microphysiological systems would become the relevant technology platform emulating the physiology and morphology of human organisms at the smallest biologically acceptable scale *in vitro* and, therefore, would enable the selection of personalized therapies for any patient at unprecedented precision. Meanwhile, the first human organoids—stem cell-derived complex three-dimensional organ models that expand and self-organize *in vitro*—have proven that *in vitro* self-assembly of minute premature human organ-like structures is feasible, once the respective stimuli of ontogenesis are provided to human stem cells. Such premature organoids can precisely reflect a number of distinct physiological and pathophysiological features of their respective counterparts in the human body. We now develop the human-on-a-chip concepts of the past into an organismoid theory. We describe the current concept and principles to create a series of organismoids—minute, mindless and emotion-free physiological *in vitro* equivalents of an individual's mature human body—by an artificially short process of morphogenetic self-assembly mimicking an individual's ontogenesis from egg cell to sexually mature organism. Subsequently, we provide the concept and principles to maintain such an individual's set of organismoids at a self-sustained functional healthy homeostasis over very long time frames *in vitro*. Principles how to perturb a subset of healthy organismoids by means of the natural or artificial induction of diseases are enrolled to emulate an individual's disease process. Finally, we discuss using such series of healthy and perturbed organismoids in predictively selecting, scheduling and dosing an individual patient's personalized therapy or medicine precisely. The potential impact of the organismoid theory on our healthcare system generally and the rapid adoption of disruptive personalized T-cell therapies particularly is highlighted.

Keywords: organismoid, organ-on-chip, microphysiological systems, real world data, immune-oncology, advanced therapies, organoid, patient-on-chip

INTRODUCTION TO THE ORGANISMOID THEORY

A human individual's lifespan is characterized by phases of development (ontogenesis) and functional maintenance (adulthood) of the physiology and morphology of the human body and a lifelong sociogenesis of an individual's soul and mind in a bidirectional person to population context (1), schematically illustrated in **Figure 1A**.

Sociogenesis is linked intrinsically to the morphological size and architecture of the human brain defined—consisting of around 86 billion neurons and a roughly equal number of non-neuronal cells (2) that are highly interconnected and clustered to process, integrate and coordinate the information it receives from the sense organs (3)—and its interconnections with the rest of the body. The physiology of the mature human body follows a simple evolutionary, selected building plan where form follows function. Back in 2007, we drew attention to the fact "[…] that almost all organs and systems are built up by multiple, identical, functionally self-reliant, structural units [...] ranging from several cell layers to a few millimeters. Due to distinguished functionality, a high degree of self-reliance and multiplicity of such structural units within the respective organ, their reactivity pattern to drugs and biologics seem representative of the whole organ. Nature created these small, but sophisticated, biological structures to realize most prominent functions of organs and systems. The multiplication of these structures within a given organ is Nature's risk-management tool to prevent the total loss of functionality during partial organ damage. In evolutionary terms, however, this concept has allowed the easy adjustment of organ size and shape to the needs of a given species (e.g., liver in mice and men), while still using almost the same master plan […]" (4). In 2012, this knowledge, combined with progress in the development of microphysiological systems (MPS), provided the basis for the first conceptual visions of emulating human bodies at the smallest biologically acceptable scale on biochips (5–7). At that time, we introduced the concept of a "man-on-a-chip" at a downscale factor of 100,000. We illustrated the functional units of the major human organs and briefly described the downscale principle (5). This was the starting point for developing a theory of the establishment of minute mindless and emotion-free physiological *in vitro* equivalents of an individual's human body, which we now call organismoids. Different terminologies, such as human-on-a-chip, body-on-a-chip, or universal physiological template, have been used in the past for organismoids, but it is common sense among the MPS community that the targeted organismal homeostasis can be achieved by combining the prime organ equivalents from at least the following 10 human systems: circulatory, endocrine, gastrointestinal, immune, integumentary, musculoskeletal, nervous, reproductive, respiratory and urinary. A chip-based system interconnecting these organ models will compose a minimal organismal equivalent and the MPS community forecasts at least another decade to establish such functional organismoids on chips (8, 9).

These can be used to emulate an individual patient's disease and healthy state, as illustrated in **Figure 1B**, therewith enabling a precise selection of the right medicine or therapy and the most efficacious exposure regime for each patient. In addition to this use for precision medicine approaches organismoids from selected cohorts of patients can further be used to conduct clinical trials on chips. Their position within the current landscape of cell models regarding their potential to emulate human physiology was illustrated in 2018 by the Investigative Toxicology Leaders Forum, which brought together representatives from 14 European pharmaceutical companies (**Figure 2**) (10).

The organismoid theory is based on two chronologically interrelated concepts, each with three principles for implementation. The concept of *in vitro* ontogenesis of an individual's organismoids relies on the principles of (i) (induced pluripotent) stem cell-based formation of premature organoids of an individual body *in vitro*; (ii) physiology-based integration of the relevant type and ratio/numbers of such premature organoids into premature self-sustained organismoids through whole blood perfusion and innervation, applying on-chip MPS; and the (iii) completion of *in vitro* ontogenesis toward healthy mature organismoids (emulating the adult stage) by organoid on-chip cross talk and accelerated exposure to ontogenic stimuli.

Subsequently, the concept of emulating the process of disease and healing of an individual patient using his/her organismoids on chips follows the principles of (i) induction of a disease in organismoids by natural disease processes or the transmission of pathogens or diseased tissues derived from the patient; (ii) the mimicry of a human clinical trial with a large number of patients by a trial with the equivalent number of healthy and diseased organismoids of one single patient; and (iii) the precise selection of the right medicine or therapy and the most efficacious exposure regime for each individual patient.

In this paper, we take you through the concepts and principles of organismoid theory, underpin the most important aspects with actual results and observations, describe its disruptive potential for our healthcare system and provide an outlook on possible approaches to a final proof of the theory.

WHAT DID STEM CELL- AND PATIENT-DERIVED ORGANOIDS TEACH US?

Deciphering the biochemical and biophysical cues leading to tissue-specific morphogenesis and organogenesis *in vivo* has fascinated scientists for over a century. Their studies were confined to classical cell culture and animal models for lack of more physiologically relevant test systems. These models have vastly enhanced our basic understanding of cellular function and disease mechanisms. However, translation of results to the human situation has become a major bottleneck. Recent

Abbreviations: 3D, three-dimensional; ASCs, adult stem cells; BBB, blood–brain barrier; CAR-T, bhimeric antigen receptor T; ESCs, embryonic stem cells; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; iPSCs, induced pluripotent stem cells; MPS, microphysiological systems; NMJs, neuromuscular junctions; PDX, patient-derived xenograft; PSCs, pluripotent stem cells; SGLT2, sodium glucose transporter 2; TEER, transepithelial electrical resistance; USD, United States dollar.

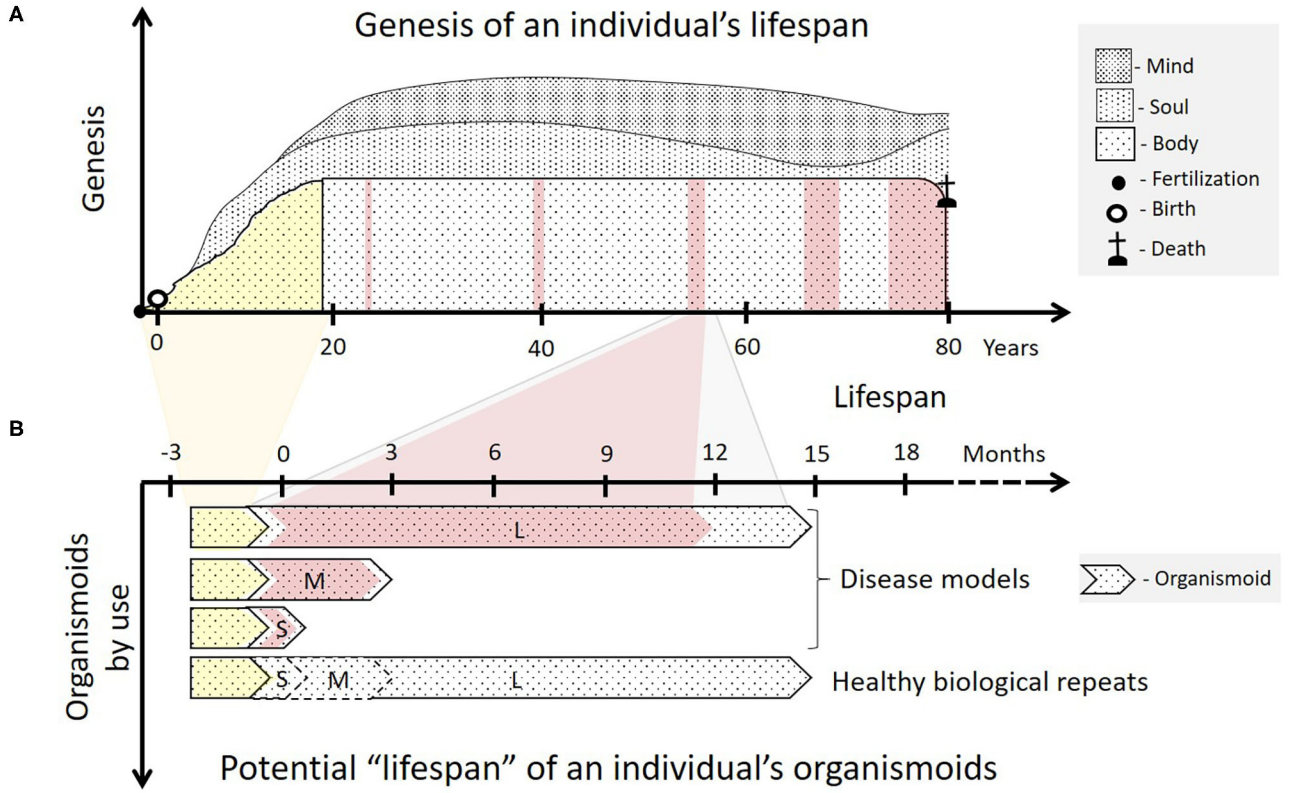

FIGURE 1 | Organismoids in the context of each human individual's fate. **(A)** The ontogeny (yellow) of an individual's body begins with ovum fertilization, followed by birth and ends with sexual maturity, a fully functional brain and an adult skeleton after 18 to 20 years. The adult body then goes through a lifelong period of relatively functional and architectural homeostasis lasting many decades. This adulthood is interrupted with increasing frequency by periods of ever-prolonging illness and recovery as the body ages (pink). Emotions and consciousness—the soul and the mind of a human being—begin to develop consecutively in childhood and continue to do so throughout life (sociogenesis). **(B)** According to the organismoid theory, personalized organismoids can be established through accelerated *in vitro* ontogenesis (yellow) lasting a few months. The resulting adult organismoids can then emulate a certain stage of healthy human adulthood for weeks (S—short-term), months (M—mid-term) or years (L—long-term), depending on use. These can then be utilized to emulate acute, sub-chronic and chronic disease periods (pink) and therapy-based recovery of an individual within the respective time frame. A large number of identical organismoids ensure that a sufficient number of healthy biological repeats can be run simultaneously serving as controls for full recovery of the diseased organismoids by a precision medicine or advanced therapy approach. Moreover, such healthy organismoids are useful to evaluate preventive medicine approaches, such as vaccination for the respective individual.

advancements in the field of stem cell research and three-dimensional (3D) culture systems have led to the generation of a promising complex and completely human model system called organoids. These organoids are generated from either pluripotent stem cells (PSCs) (either induced PSCs [iPSCs] or embryonic stem cells), adult stem cells (ASCs) or adult tumor tissue by self-organization. Their use for drug discovery and personalized medicine has been reviewed (11) and first proof of concept to generate personalized data has been provided (12).

Organoid tissues maintain their capacity to keep proliferating and differentiating into the different cell types of the respective organ, while preserving a stem cell pool, by carefully tuning microenvironmental cues, such as mimicking the *in vivo* stem cell niche. In comparison to two-dimensional monolayer cultures, 3D organoid cultures mimic more closely the physiological behavior of organs shown by gene and protein expression and metabolization capacity. The majority of organoids require an extracellular matrix environment, which is based on laminins and collagen, comparable to the physical scaffold surrounding cells *in vivo*. This matrix is, in most cases, animal-based and not well-defined, therefore, batch-to-batch variabilities might occur (13). Furthermore, the differentiation process of organoids, ASC- or PSC-derived, depends on many different elements, such as growth factors, the matrix, matrix stiffness, cell-cell contact, cell density, oxygen level, nutrient supply or the stochastic nature of *in vitro* self-organization and cell fate decision. Thus, there is a high heterogeneity in the maturation and function of organoids under standard *in vitro* culture conditions.

Organoids derived from PSCs mimic embryonic development *in vitro*. Therewith, these organoids are of great value for developmental studies. Different growth factors are used to push the PSCs into the appropriate germ layer—mesoderm, endoderm, or ectoderm. Subsequently, further growth factor cocktails are used to drive the cells to form a differentiated organoid. Here, matrix proteins play a crucial role in organoid formation and are frequently used to mimic the basal lamina.

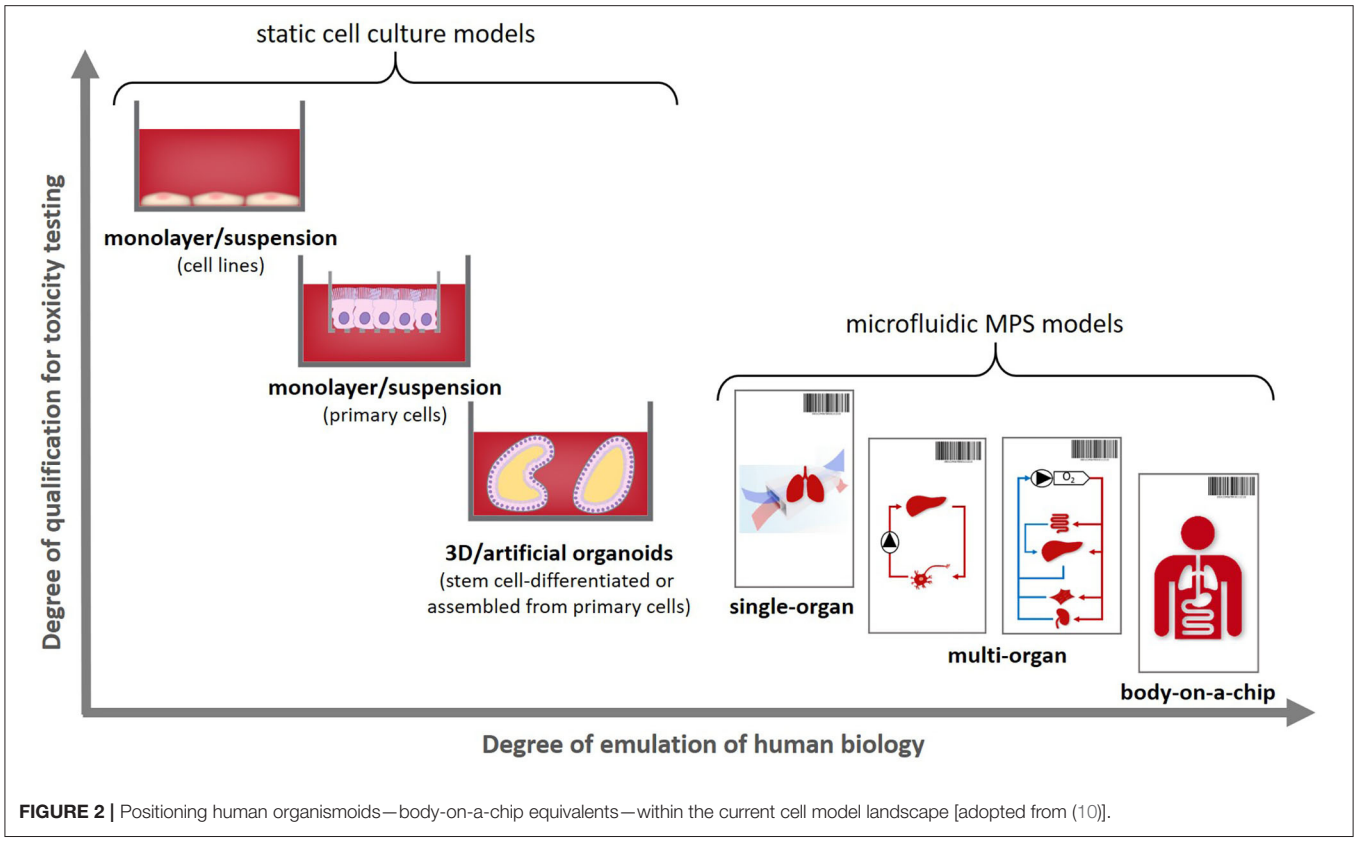

FIGURE 2 | Positioning human organismoids—body-on-a-chip equivalents—within the current cell model landscape [adopted from (10)].

The differentiated organoids may consist of different cells types—epithelial and mesenchymal—and may even acquire initial endothelial networks by the intrinsic differentiation (14) or extrinsic addition of endothelial cells or mesodermal progenitors (15, 16). The period of generating PSC-derived organoids varies depending on the tissue type and usually requires between 2 weeks and 3 months (17) but can continue for half a year or longer, as seen in skin (18) or brain organoids (19).

Patient-specific PSC-derived organoids are generated by reprogramming somatic cells into iPSCs. However, this may take several months. The efficiency of organoid differentiation varies greatly between tissue types and even differentiation protocols. Furthermore, there is a limited possibility for the passaging of iPSC-derived organoids.

Organoids are difficult to generate from ASCs for some tissues, such as the brain, due to a lack of availability of tissue samples. Therefore, PSC-derived organoids are beneficial for brain organoid generation. These organoids have been cultured for up to and beyond 2 years in 3D spheroid suspension culture without passaging (19, 20).

Organoids derived from ASCs are generated from adult tissue having regenerative ability. Early human tooth or hair follicle development models, for example, apply mesenchymal condensation principles *in vitro* to generate the placode or the dermal papilla organoids, respectively from donor-derived progenitor cells (21, 22). However, only the epithelial portion of tissues can be made into organoids. Stromal cells, endothelial cells and nerves are missing in these models. The major benefits of ASC-derived organoids lie in the fact that they may be generated from healthy or tumor tissue. Organoid formation of ASCs normally takes only several days and the organoids are stable for long-term cultivation and expansion. Distinct growth factor cocktails are used for organoid expansion and differentiation, therefore, they can be expanded indefinitely. Biobanks of healthy and tumor organoids from patients can be generated from different organs to test drugs in high throughput screenings for further decision-making for patient treatment (17).

A large variety of human organoids has been generated using these 3D cultivation methods in academic labs over the past decade. Their applications and their potentials have been extensively reviewed (13, 17, 23–25). **Table 1** highlights the human organs for which organoids have been generated in static *in vitro* culture. However, conventional static culture systems cannot terminally differentiate the organoids into mature and fully functional organ models. Local morphogen gradients are appearing in organoids as they are forming but stable blood perfusion-driven morphogen gradients of growth factors, oxygen and other functional biochemical or biophysical cues are missing. Thus, static culture conditions limit the cultivation time due to restrictions in the supply of nutrients and waste removal from ever-growing organoids, therewith limiting their maturation grade. In the following, we will discuss how to improve organoid maturity by introducing defined spatiotemporal cues.

TABLE 1 | Overview of human organoids generated in static *in vitro* culture.

Organ	Tissue source	Culture condition	Functionality or application	References
Blood vessel	iPSCs, epithelial stem cells (ESCs)	Spheroids in ultralow attachment plates or matrix-embedded	Endothelial cells and pericytes that self-assemble into capillary networks	(26)
Brain	iPSCs, ESCs	Suspension spheroids or matrix-embedded	Unpatterned organoids contain cell clusters with forebrain, midbrain, hindbrain and retinal identities that contain glutamatergic, GABAergic and dopaminergic neurons as well as astroglia. Patterned organoids can be differentiated toward forebrain, midbrain, brainstem, cerebellum, thalamus, hypothalamus, spinal cord and hippocampus identity	(19, 27–42)
Liver	ASCs, ESCs, iPSCs	Suspension spheroids or matrix-embedded	Appropriate secretion ability (albumin and urea) and drug metabolic ability (CYP3A4 activity and inducibility)	(43–56)
Thyroid	Adult thyroid-derived cells	Matrix-embedded	Secretion of thyroid hormones	(57)
Pancreas	ASCs, ESCs, iPSCs	Matrix-embedded	Secretion of insulin in response to glucose	(58–60)
Optic cup	iPSCs, ESCs	Matrix-embedded	Primitive cornea and lens-like cells, developing photoreceptors, retinal pigment epithelia, axon-like projections and electrically active neuronal networks	(61–66)
Intestine	ASCs, ESCs, iPSCs	Matrix-embedded	Villus- and crypt-like structures and enterocytes, goblet, enteroendocrine, and Paneth cells	(67–71)
Gastric	ASCs, iPSCs	Matrix-embedded	Used to study H pylori infection and other gastric pathologies	(72–75)
Kidney	ASCs, ESCs, iPSCs	Spheroids or matrix-embedded	Rudimentary nephrons, and 3D culture combined with active fluid flow	(76–81)
Lung	ESCs, iPSCs, fetal lung tissue, ASCs	Matrix-embedded	Rudimentary bronchiole-like structures and express alveolar cell markers	(82–87)
Skin	iPSCs	Aggregates in suspension with matrix coating, skin Transwell model	Complex skin analogs with human iPSC (hiPSC)-derived keratinocytes, endothelial cells and fibroblasts	(18, 88, 89)
Cardio	iPSCs, ESCs	Spheroids or monolayer	Contractile spheroids	(90–94)
Mammary gland	adult mammary gland tissue	In adherent or floating matrix	Could be induced to produce milk protein	(95, 96)
Prostate	ASCs	Matrix-embedded	With basal and luminal cells	(97–99)

MICROFLUIDIC CELL CULTURE SYSTEMS—THE KEY TOWARD THE INTEGRATION OF PREMATURE ORGANOIDS INTO ORGANISMOIDS

Organoids have proven to be powerful tools in emulating distinct sets of organ-specific characteristics. However, marker expression and functionality often halts at a premature stage, as described above. We have known since 1912 that the environment of *in vitro* cultures defines their viability and functionality (100). The isotropic microenvironmental cues that have driven organoid self-assembly and differentiation engulf organoids under traditional culture conditions rather homogenously or cover extensive surface areas hindering the spatial orientation and maturation driven by functionality. But these spatiotemporal cues originating from interacting tissues and leading to a rearrangement of cells are key to the development of mature organ functionality. Endothelial-tissue cross talk in particular and its implications for local signaling during organogenesis have been studied extensively (101–103). Vascularization of the developing central nervous system, for example, is a crucial step in brain development ensuring oxygen and nutrient supply of the rapidly dividing neural progenitors. Neural structures of the peripheral nervous system have been demonstrated to develop in noticeable alignment with blood vessels. Furthermore, the importance of endothelial cells for the maintenance of the germinal zones of the central nervous system where cerebellar cells are produced has been shown (104).

Moreover, the recombinant proteins and small molecules administered in static cultures to control lineage specification mostly promote the development of a specific subsection of cells within the organ (the parenchymal cells). Other crucial lineages, such as vascular, neuronal or immune lineages, are mostly absent, therewith silencing paracrine signaling that might become relevant during further maturation.

Allowing a finely orchestrated systemic interaction of premature organoids with other organ systems at physiologically relevant scales promotes an alignment of functionality and a higher spatial resolution of stimulation. Nature's organ building

blocks—the smallest functional units described above—form, in their multiplicity, an entire human organ. The number of those repetitive subunits depends on the requirements signaled by the interacting organs. Therewith, organ sizes, medium flow rates and fluid residence times in organs and overall liquid to cell ratios self-adjust in a dynamic interplay of tissues.

Several approaches enabling the systemic interaction of tissue models have been devised to date—the most prominent being patient-derived xenograft (PDX) models and MPS. In the former, immunodeficient or humanized mice serve as hosts, enabling the engraftment of primarily tumor models. The interplay of grafts with local and systemic environments, also through a vascularization of the models, eventually allows for a nutrition of cells and a propagation of models. However, species' differences between the host organism and the patient's tissue prevents a complete match of biology. A plethora of drawbacks have been described in using these methods, but the assets of having a systemic circulation supporting the models could be shown.

Substantial efforts have been made over the past two decades to improve organ model culture conditions by introducing them into MPS. Dozens of human organ equivalents in MPS have been established using primary- and cell line-based models and have been reviewed in great detail (105–111). It is well-documented that the maturation of organ function can be achieved by closely emulating organotypic microenvironments regarding biochemical, physical, or electrical stimuli (106).

Once the notion became clear that the autologous nature of such systems is essential, the MPS community started to establish stem cell-derived models on-chip. **Table 2** summarizes the very recent achievements in this area. Furthermore, it is only a matter of time before the missing organoids for the creation of minimal organismoids are established.

A huge variety of additional human tissue and organ models have been published. However, attempts at organismal on-chip homeostasis have so far failed to integrate the systemic components, such as whole blood supply through vascularized microvessels, a personalized (autologous) immune system and tissue innervation. Here, we have extended the theorization of creating MPS-based organismoids by integrating the premature organoids of each relevant organ system into a self-regulating vascularized and innervated systemic circulation.

We hypothesized already in 2012 that "the lack of a dynamic interplay between organ-specific cell types, with their vascular and stromal tissue bed, and the absence of adult stem cell and progenitor niches for local regeneration, are responsible for the crucial missing capabilities of current 'human-on-a-chip' systems" (5). The vascularization of whole microfluidic circuits on-chip was shown as early as 2013 (132), followed by fascinating work on the generation of vascularized organ models on-chip (133–136). As of today, high-throughput platforms generating vascularized single-tissue models on-chip are commercially available (137). The combination of both technologies generating a closed vascularized circuit containing multiple organs on-chip is within reach, allowing for the next level of physiological complexity—the perfusion of whole blood or a defined substitute containing all relevant components.

The organismoid theory hypothesizes that the generation and renewal of all crucial whole blood components—red blood cells, platelets, white blood cells and plasma components—is feasible and will lead to a self-sustained systemic organismoid. Therefore, a steady functional on-chip hematopoiesis is required. Several approaches to model the human bone marrow-based hematopoietic stem cell niche using MPS have been described (8, 138, 139). Adapting the model of Sieber et al. (138) to include cytokines important for cell differentiation and stem cell maintenance has enabled the continuous, robust generation and maintenance of cells from erythroid, myeloid and megakaryocyte lineages, while simultaneously maintaining stem and progenitor cell populations for at least 24 days (**Figure 3**). In brief, bone marrow chips were established as described by Sieber et al. (138) but with the modification of media to include additional cytokines, as outlined in Chou et al. (139). Cells were sampled from the recirculating media and deposited directly onto slides using a cytospin centrifuge, before staining with Wright's stain and imaging. The donor information for the cells used in the study is detailed in **Table 3**.

The academic MPS development landscape has provided a number of other indicators that particular elements of blood perfusion can be recapitulated using MPS. The capability of emulating platelet-induced blood coagulation has been demonstrated by Westein et al. (140) and numerous publications describe the circulation of immune cells in MPS and their settlement in organ models on chips (141–143).

As soon as it comes to organismoid-based physiological whole blood provision to all on-chip organ equivalents, the assets of using stem cell-derived organoids of an autologous source becomes relevant to prevent foreign organ model rejection by the immune system. Multi-organ systems published previously were mostly composed of tissues from different donors, which made the rejection-free integration of an individual's immune system, as the major defense mechanisms of any human organism, impossible. The first steps toward an autologous co-culture of several cell types from one iPSC donor were reported as early as 2013 (144). The premature nature of iPSC-derived organoids raised the question of how such organoids can be finally differentiated to match the functionality of their respective human counterparts. Here, the organismoid theory proposes the principle of terminal on-chip differentiation, guided by integrated organismal cross talk and artificially accelerated "training programs" for key organs and systems, such as xenobiotic panel exposure for the liver, multi-antigen vaccination for the immune system or artificial exposure to steroid hormones for the accelerated maturation of the sexual organs. The first hint that further on-chip maturation is triggered by organ-organ interaction in a physiology-based 4-organ chip was demonstrated in 2019 (131), where the expression of albumin and MRP2 genes increased significantly over a period of 14 days in an iPSC-derived premature liver model, driven solely by differentiation factor-free co-culture with iPSC-derived intestinal, kidney and neuronal models (**Figure 4**).

Such data support the organismoid theory's concept that once liver functionality matches the requirements of the systemic organismoid, a regular application of nutrients through the

TABLE 2 | Examples of the MPS-based models established recapitulating functions of the key human organs.

Organ	Substructure/Cell types	Tissue source	(Patho-) Physiology	Chip type	MPS advantage	References
Brain	Unpatterned (forebrain + hindbrain)	hiPSCs	Healthy, + prenatal nicotine exposure	Continuous unidirectional perfusion	Enhanced expression of cortical layer markers (TBR1 and CTIP2) under perfusion	(112, 113)
	Unpatterned (forebrain + midbrain + hindbrain)	hESCs	Healthy	Continuous unidirectional perfusion	Creation of signaling gradients that mimic developmental patterning for neural tube formation	(114)
	Blood-brain barrier (BBB; endothelial cells + motor neurons)	iPSCs	Healthy	Continuous unidirectional perfusion	Increased calcium transient function and chip-specific gene expression under perfusion	(115)
	BBB (endothelial cells + neural progenitor)	iPSCs	Healthy + diseased (Huntington's disease)	Continuous unidirectional perfusion	Physiologically relevant TEER and BBB permeability, capillary wall protected neural cells from plasma-induced toxicity	(116)
	GABAergic neurons and astrocytes	iPSCs	Healthy	Continuous bidirectional perfusion		(117)
Optic Cup	Retina	hiPSCs	Healthy	Continuous unidirectional perfusion	Recapitulation of the interaction of mature photoreceptors with retinal pigment epithelium	(118)
Liver	Hepato- and cholangiocytes	hiPSCs	Healthy	Continuous unidirectional perfusion	Improved cell viability, higher expression of endodermal mature hepatic genes and improved functionality under perfusion	(119)
	Hepatocytes	hiPSCs	Healthy	Continuous unidirectional perfusion	Higher potential hepatic progenitor cells to hepatic organoids under perfusion	(120)
Pancreas	Islet-specific α- and β-like cells	hiPSCs	Healthy	Continuous unidirectional perfusion	Enhanced expression of pancreatic β-cell gene and protein expression and increased β-cell hormone production under perfusion	(121)
Heart	Cardio-myocytes	hiPSCs	Healthy + diseased (Barth syndrome)	Continuous unidirectional perfusion	Enabled description of metabolic, structural and functional abnormalities associated with Barth syndrome	(122)
	Cardio-myocytes + endothelial cells	hiPSCs	Healthy	Continuous unidirectional perfusion	Endothelial cells align with the flow and form tube-like networks in the cardiac muscle channel	(123)
Intestine	Duodenum	Human ASCs (tissue biopsies)	Healthy	Continuous unidirectional perfusion	Human-relevant functionality is superior to that of organoids alone	(124)
	Unpatterned	hiPSCs	Healthy	Continuous unidirectional perfusion	Polarized, contains all the intestinal epithelial subtypes and is biologically responsive to exogenous stimuli	(125)
	Small intestine	Human ASCs (tissue biopsies)	Healthy	Repeated unidirectional perfusion	Removal of dead cells from the organoid tubes under perfusion allowed long-term culture > 1 month	(126)
	Unpatterned	hiPSCs	Healthy	Continuous unidirectional perfusion	Luminal waste removal through continuous flow	(127)
Stomach	Gastric organoids	hiPSCs	Healthy	Continuous closed loop perfusion	Rhythmical stretch and contraction—reminiscent of gastric motility	(128)
Kidney	Glomerolus (podocytes)	hiPSCs	Healthy	Continuous unidirectional perfusion	Differential clearance of albumin and inulin when co-cultured with human glomerular endothelial cells	(129)
	Glomerolus + tubulus organoid	hiPSCs	Healthy	Continuous unidirectional perfusion	Generation of perfusable vascular networks and better cell maturation under perfusion	(130)
Multi-organ	Brain, intestine, kidney, liver	hiPSCs	Healthy	Continuous circular perfusion	Co-culture over 14 days in one common medium deprived of tissue-specific growth factors	(131)

intestinal model and removal of waste substances through the kidney model will suffice to maintain functional homeostasis of the organismoid.

The majority of plasma proteins in humans are produced by the liver with albumin at a concentration of about 40 g/L, plasma being by far the largest component. In addition, the liver

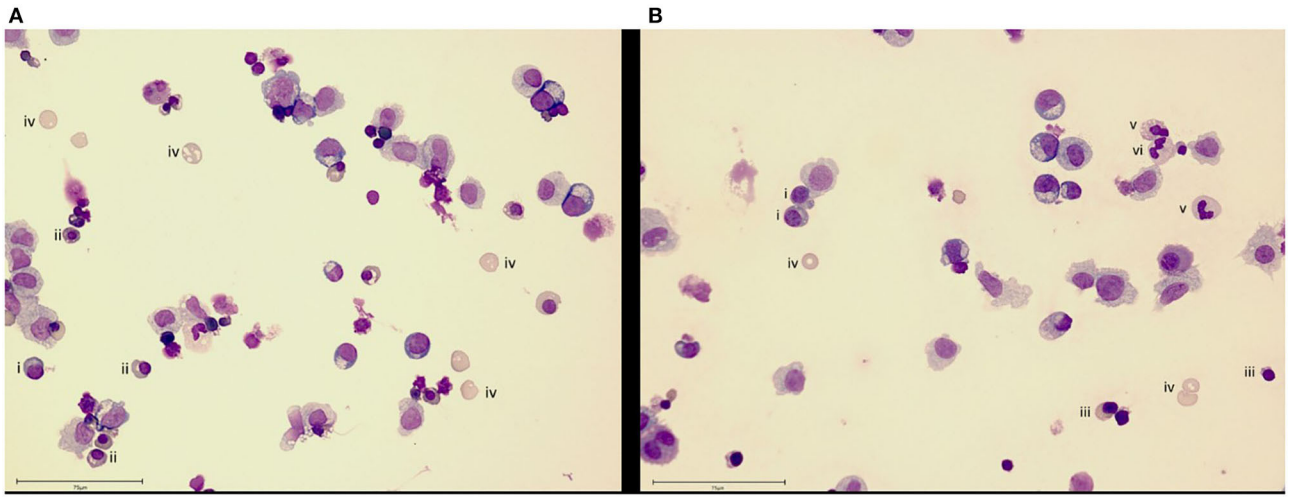

FIGURE 3 | Bone marrow cell maintenance and differentiation in an MPS. Culture of human CD34+ cells on a human mesenchymal stem cell-seeded scaffold in the recirculating HUMIMIC Chip2 for **(A)** 17 or **(B)** 24 days shows differentiation into erythroid (day 17) and then, additionally, neutrophil (day 24) lineage cells. Cells are identified as (i) basophilic normoblast, (ii) polychromatic normoblast, (iii) orthochromatic normoblast, (iv) reticulocyte, (v) band cell and (vi) neutrophil. Scale bar—75 μm.

TABLE 3 | Stem cell donor information for the bone marrow MPS.

Human Mesenchymal Stem Cells (MSC)				**Human CD34+ Hematopoietic Stem Cells (HSC)**			
Donor (Lot #)	**Sex**	**Age**	**Race**	**Donor (Lot #)**	**Sex**	**Age**	**Race**
0000451491	Male	25	Caucasian	0000680575	Female	21	Black

is a gatekeeper for toxicants and xenobiotics arriving through the food. Finally, it keeps homeostatic conditions regarding protein, carbohydrate and amino acid metabolism throughout the circulation. A plethora of MPS literature has provided evidence that human liver equivalents on chips composed of primary or iPSC-derived hepatocytes are capable of continuously secreting albumin and other proteins into the circulation (119). Another important protein component is the immunoglobulin G fraction produced by white blood plasma cells, reaching physiological levels of 7–16 g/L in the plasma protein fraction in humans. Very few MPS have aimed to generate immunoglobulins in modeling immune tissues, but an artificial lymph node has made initial progress here (145). Some other organs add crucial regulatory proteins to the plasma. Insulin secreted by the pancreatic islets and interacting with the liver to control the body's glucose-based energy balance through the regulation of glucose consumption, storage and release is such a key regulator. A stable pancreatic islet–liver co-culture MPS demonstrating the capability of an MPS to physiologically manage the secretion and organ interaction capacity for insulin was established in 2017 (146). Regarding plasma properties, it still remains a challenge to increase the plasma protein concentration generated on-chip toward physiological levels of 60–80 g/L, which is a factor of outstanding importance to leverage the physiological transport properties of albumin, deliver the hundreds of other functional proteins to their sites of action, and provide the right viscosity and flow dynamics for the transport of blood cells across the organism. The complete separation of the organ models from the blood flow by endothelial cells is a basic prerequisite for this and will provide progress in that field in the near future.

A preliminary adjustment of the plasma composition by technical means is feasible by closely monitoring metabolic activities on-chip by online sensors and might lead to an advanced maturation of organ models in the long term. Here, the use of automated systems maintaining a close observation of on-chip cultures will become an essential component (147).

Sensors can generally play a crucial role in the implementation of the organismoid theory, extending an individual's data generation beyond any currently available. On the one hand, the sensors can be inherent in the system. Analogous to pulse oximetry, for example, the oxygen saturation in the blood of an organismoid can be measured using different optical spectra of the various hemoglobin derivatives. In addition, pulse oximetry provides qualitative information about the pulsatile properties of the blood (148). The measurement of the oxygen saturation can be performed using miniaturized sensors or spatially resolved by using hyperspectral imaging techniques. The measurement of the oxygen saturation provides important information about the oxygen transport capacity, oxygen distribution and, in combination with microparticle imaging velocimetry analysis, the absolute oxygen consumption rate of each organoid incorporated into an organismoid (149).

On the other hand, the sensors can be embedded directly into the MPS. In parallel with the body area network

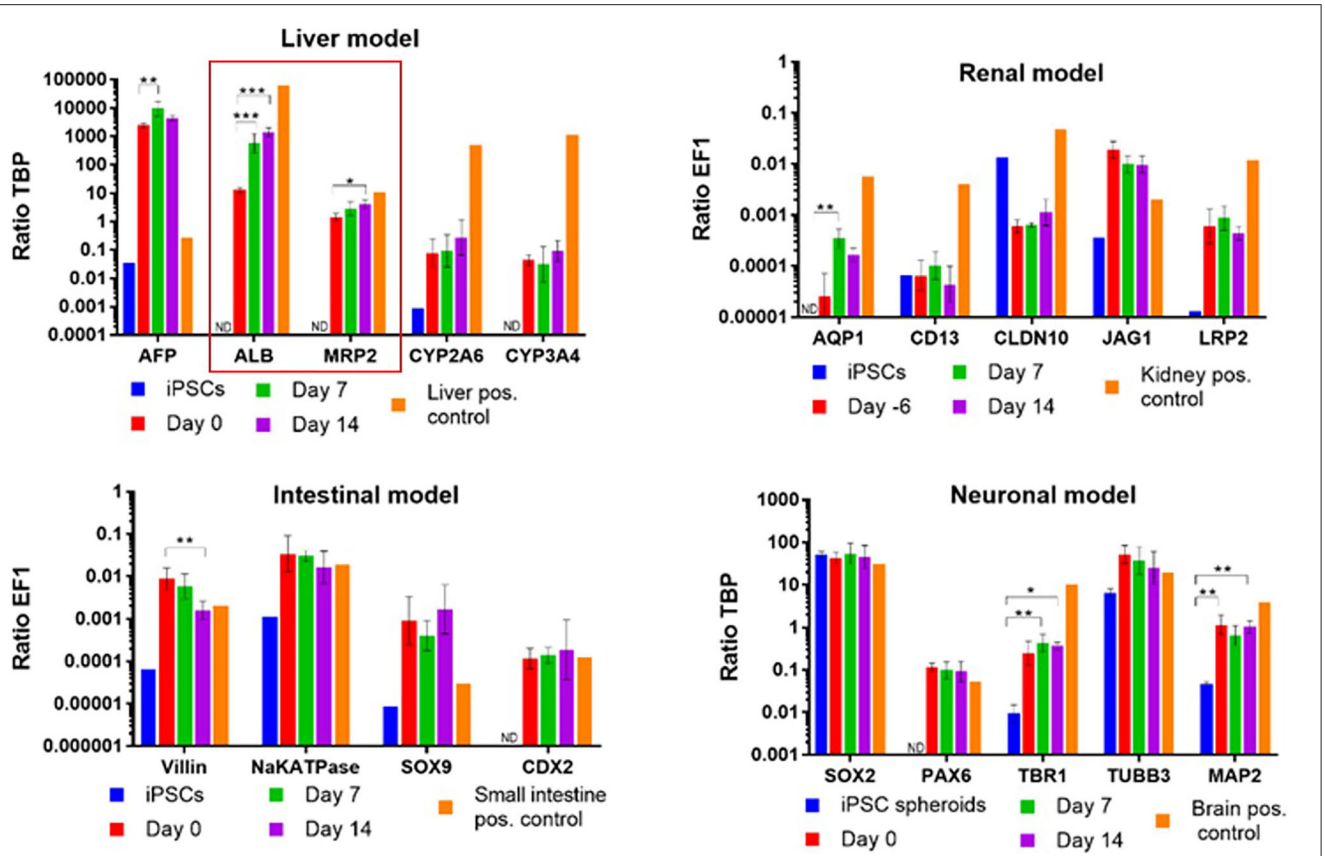

FIGURE 4 | Albumin and MDR2 gene expression increased steadily from day 0 to 14 in a 4-organ chip composed of iPSC-derived premature intestinal, liver, kidney, and neuronal organ models integrated in a physiology-based common media circulation. Feeding of differentiation factor-free medium solely through the intestinal model was performed. One-way ANOVA with Tukey's multiple comparisons test was used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data shown as Mean + SD.

technology for the health monitoring of the human body (150), the integration of multiple sensors is fundamental to allow the continuous online monitoring of organ-specific reactions and dynamic tissue responses. Multi-sensor integrated platforms are especially important for the development of MPS-based organismoids in which the monitoring of function of various organs requires a combination of different sensing principles. Transepithelial electrical resistance measurement is among the most popular non-invasive techniques and has been successfully integrated into MPS to assess the barrier integrity and junction dynamics of endothelial or epithelial models (151). Electrical impedance spectroscopy-based methods overcome conventional transepithelial electrical resistance measurement techniques by exploiting extended frequency domain data, thus, allowing an evaluation of tissue barrier function at different maturation stages (152). Impedance-based techniques can be further enhanced when coupled with multi-electrode arrays to provide localized sensing and electrical stimulation in relevant microenvironments of an organismoid. Applications include the recapitulation of cardiomyocyte or motor neuron innervation by direct electrical stimulation of the contractile activity (153), and the possibility of producing surrogate electroencephalograms from neuronal activities, which represents an added value to Parkinson or Alzheimer's disease modeling.

The innervation of organ models plays a pivotal role in their development, maturation, regulatory control, regeneration and pathology. We know from organ transplant surgeries that the ablation of autonomic neuronal connections may cause poor graft functionality and detrimental health effects (154). Similarly, the importance of innervation in the regulation of stem cells and/or their niches in most organs and tissues has been well-documented. Autonomic nerves impact tissue growth during the initial organogenesis and regeneration and, similarly, impact aging or the development and progression of disease. The introduction of innervation to *in vitro* models has so far been mostly neglected due to the complexity of achieving proper guidance and integration of neurons into non-neural tissue models. First steps have been taken to model both the synaptic junction between neural models and the neuroeffector junction formation between neural and non-neural tissue models both in static and MPS organoid cultures during the last decade. A major challenge in this field is the directed guidance of axon growth from the neuronal to the effector tissue. Consequently, the development of MPS that enable the assembly and cultivation of stem cell-derived myelinated motor neurons,

as published by (155, 156), are an important basis for enabling functional innervation in MPS. Understanding how tissue-derived neurotrophic and neural guidance factors drive axon growth and determine its directionality during development will be instrumental.

Stem cell-derived regionalized brain organoids have been shown to possess the intrinsic capability of forming synaptic innervations with each other. Multiple groups have described the fusion of regionalized stem cell-derived cortical and subpallium organoids to model the migration of GABAergic interneurons from the subpallium organoids into the glutamatergic excitatory, neuron-rich cortical organoids, where they integrate functionally into local excitatory circuits (157–159). This intrinsic capability can also be exploited for the assembly of the other regionalized brain organoids, as has been shown for stem cell-derived cortical and thalamic organoids (159).

Roger D. Kamm's group has shown that stem cell-derived motor neuron organoids can build functional neuromuscular junctions (NMJs) with 3D skeletal muscle bundles in a patterned chip platform which enhanced the guided innervation of tissue constructs. Motor neurons in this setup were transduced with the light-sensitive channel rhodopsin-2, which enabled light-activated muscle contraction to show the formation of functional NMJs (160). Another study with stem cell-derived cerebral organoids showed that they could develop pronounced axon tracts, which functionally innervated into rodent spinal cord explants where they caused concerted muscle contractions easily distinguishable from local spontaneous contractions and could be evoked by electrical stimulation (31). The positive effects of nerve innervation on the maturity and functionally of cardiac tissue was shown by (161), where the innervation of sympathetic neurons increased the spontaneous beat rate of primary cardiac cells.

Protocols have been developed to omit the complex guidance of axon growth to the respective non-neural tissue organoid; these allowed the simultaneous differentiation of neural and non-neural tissue in one organoid. This has been achieved for neuromuscular organoids that contain functional NMJs and myelinated axons in the presence of terminal Schwann cells and contractile activity of the muscle part, which stopped upon blockage of acetylcholine receptors (162). Such interorgan spheroids were also realized for a combination of forebrain and optic vesicles, where bilateral light-sensitive optic vesicles developed on the surface of forebrain organoids and formed electrically active primitive sensory circuits (62).

The next big steps in the field of *in vitro* tissue innervation will be to build a closed neuronal circuit model with a sensory (e.g., optic cup organoid) and effector (e.g., muscle fiber) that are interconnected by a cortical model, and achieving the myelination of motor neurons axons by Schwann cells or oligodendrocytes (156, 163). Another promising approach is the combination of on-chip vascularization and innervation. *In vivo* peripheral nerves grow along blood vessels. We hypothesize that this route of innervation will also become relevant on-chip once the closed vascular system is established. The advantages of the interconnection of vascularization and innervation were shown by (116), who demonstrated vascular-neural interaction leading to a more *in vivo*-like gene expression signature and increased calcium transient in a MPS equipped with stem cell-derived brain microvascular endothelial cells and motor neurons.

In addition to the positive effects of physiological tissue innervation by (motor-)neurons, modeling of the peripheral nervous system is also of interest for the field of neurodegenerative diseases such as amyotrophic lateral sclerosis. *In vitro* (MPS) models have the potential to become an important cornerstone for studying the pharmacological effects of compounds on the NMJs. A first step in this direction was recently published by (164) on the Mimetas OrganoPlate platform, which hosts 40 microchips with iPSC-derived motor neurons. The latter showed pronounced axon outgrowth and could be coupled to muscle tissue to form NMJs.

Another critical aspect regarding organismal homeostasis is the interaction of autonomic innervation with the immune system. Looking at the digestive tract, for example, the gut immune system impacts on the local enteric nervous system, the extrinsic neurons of sympathetic and parasympathetic systems and, ultimately, on brain functions, such as mood, cognition and mental health. Conversely, the brain is able to modulate immune function in the intestine through the vagus nerve via the intestinal cholinergic anti-inflammatory pathway (165). Innervation in primary lymphoid organs, such as the bone marrow, and secondary lymphoid organs, such as the spleen, has been well-studied and the ability of the nervous system to influence immune homeostasis and inflammation in these niches has been shown (166).

Another important aspect to emulate systemic organismal pathways on chips includes the integration of the relevant donor-specific microbiota to mimic a patient's interaction with the respective metabolites in general (167–169).

It appears that innervation, vascularization, lymphatics, microbiota, and the emulation of a human-like enterohepatic circulation of bile products are indispensable prerequisites to bridge the gap between the simple physical combination of organoids in multi-organ MPS and real tissue interaction and homeostasis in an organismoid.

The latter needs a biological combination of the prime organ equivalents from at least 10 human systems (as highlighted in the introduction) and their biological interconnection through vasculature, innervation and lymphatics. Two early attempts to establish MPS containing at least 10 technically interconnectable organ culture compartments have already been published. Those prime examples include the 13-organ culture compartment system of the Shuler Lab at Cornell University (170) and the 10-organ culture compartment PhysioMimix^{TM} system of the Griffith lab at MIT (171). Both systems have been successfully operated with biological materials in the culture compartments for seven or more days. However, both lack a biological blood vessel interconnection, lymphatics and organ innervation.

WHAT ORGANISMOIDS MIGHT DELIVER TO OUR HEALTHCARE SYSTEM

According to the organismoid theory, organismoids are biological replica of the living human body *in vitro*, reduced in scale as far as possible. They are created by the systemic

physiological integration of the functional units of the major human organs into an organismal, self-sustained template that reflects the systemic organization of the human body. The on-chip fast-track differentiation of stem cell derived organ equivalents originates from their cross talk and a physiological reliance on each other. The extreme reduction in scale is due to the goal of generating a large number of replicates of the organismoid of an individual. Large numbers of such identical, minute, mindless and emotion-free physiological *in vitro* equivalents of an individual's mature human body can be maintained at self-sustained functional healthy homeostasis over very lengthy time frames. They are open to perturbation leading to the natural or artificial induction of diseases. The diseased organismoids are hypothesized to emulate the pathophysiology of the respective patient's disease precisely. This, in turn, may enable the performance of predictive patient-specific organismoid studies to determine the most effective personalized therapy for the patient concerned. Similar to clinical studies on patient cohorts, statistically verified predictions can then be generated with the advantage that genetically identical replicates of the patient's organismoids can be compared under physiological and pathophysiological conditions. Two major usage scenarios can be derived from that. One is related to a cutting-edge improvement of an individual patient's personal treatment in the real world; the other has the potential to change the drug development paradigm on a clinical trial level, saving enormous amounts of time and capital expenditure.

Regarding the first scenario, organismoids can be used in predictively selecting, scheduling and dosing an individual patient's personalized therapy or medicine accurately along their disease progression. This can significantly decrease the potential risk to each and every patient by the early detection of unsuccessful treatment schedules. **Figure 5** summarizes the advantages of applying organismoids for personalized precision medicine in more detail. The figure illustrates the concept and principles of the organismoid approach to select the best fitting precision medicine applied to your personalized illness. As a hypothetical example, cancer is chosen to be the illness.

Your lifespan might eventually include periods of life-threatening illness, for example, cancer growth (top: Blue-bordered arrow). A pluripotent stem cell bank is established from your healthy cells. Subsequently, a large number of identical healthy organismoids are generated within a few months (yellow triangle). Various options are currently available to treat cancer, therefore, relevant test groups are created, including placebo treatments, other treatment groups and healthy recovery controls (in the black-bordered arrow). In this hypothetical example, within a few weeks, the CAR-T cell therapy in combination with a checkpoint inhibitor turns out to be the fastest and most effective cure for you. Therefore, this therapy is immediately and successfully applied.

According to the organismoid theory, an individual's stem cell bank can be created when healthy or from a healthy organ when illness occurs. Preventive stem cell banking (e.g., from umbilical cord blood) is already in use and will be the choice in the future as it takes time. The near-to-human element of the theory provides precise test results which animal tests in patient-derived xenograft models or human patient-derived organoids cannot achieve. Xenograft models are phylogenetically distant and, therefore, cannot provide sufficient tumor outgrowth. Additionally, they do not have a patient's immune background to fight cancer. Patient-derived organoids are also not embedded in the patient's immune system and lack systemic interaction with the organism.

Regarding the second scenario, the average success rate of drug candidates entering clinical trials to become an approved drug has been below 20% for decades; no other industry can afford this inefficiency of translating any prototype into a marketed product. Poor predictivity of the preclinical safety and efficacy evaluation program of candidate drugs using laboratory animals is the prime reason for that inefficiency. Lengthy clinical trials averaging 13.5 years and bearing cumulative costs reaching as much as 2.5 billion USD to get a new medicine approved are the consequences (106). Simultaneously, a revolution in therapeutic strategies has emerged over the last three decades based on biologics—using the human body's own tools to fight diseases. The expanding biological complexity of medicines, from synthetic low molecular weight drugs toward, for example, human monoclonal antibody proteins and, finally, patient-specific autologous cell therapies, has dramatically increased the chances of cure for patients in recent years. However, this trend has, just as dramatically, reduced the chances of being able to predict the safety and efficacy of such therapies by applying preclinical laboratory animal testing due to the increasingly human origin of such advanced therapeutic medicinal products (172). In addition, the pricing along this rising biological gradient of medicines has become the major roadblock for the socially equitable availability of such therapies for all patients in the last few years. At the beginning of that trend, the average daily dose of a biologic drug cost 22 times more than that of a small molecule and accounted for a few dozen USD (173). However, best in class protein biologics—monoclonal antibodies—reached an annual average price for a patient's therapy course of about 96,000 USD in 2017 (174), which corresponds to roughly 263 USD per day. Nowadays, the price for the most disruptive innovation in advanced cell therapies—highly effective autologous CAR-T cell therapies—in Germany, for example, rose to as much as 320,000 € for a patient's treatment, considering a payment "at" result (175). This therapy is a single day infusion. An ever growing misbalance between the efficacy of wonder-performing drugs and the patient's financial ability to access them has become a serious social and economic conflict for our healthcare systems on a global scale.

Organismoids have the potential to break this cost spiral by bringing about a paradigm shift in drug development. The stakeholder report of the MPS community produced in 2016 already projected a decrease of the cumulative drug development costs by a factor of five and a halving of the drug development times once MPS-based clinical trial-like studies on organismoids have enabled the accurate prediction of efficacy, safety, dosing and scheduling for any new medicine or therapy prior to use in human and replacing animal testing and Phase

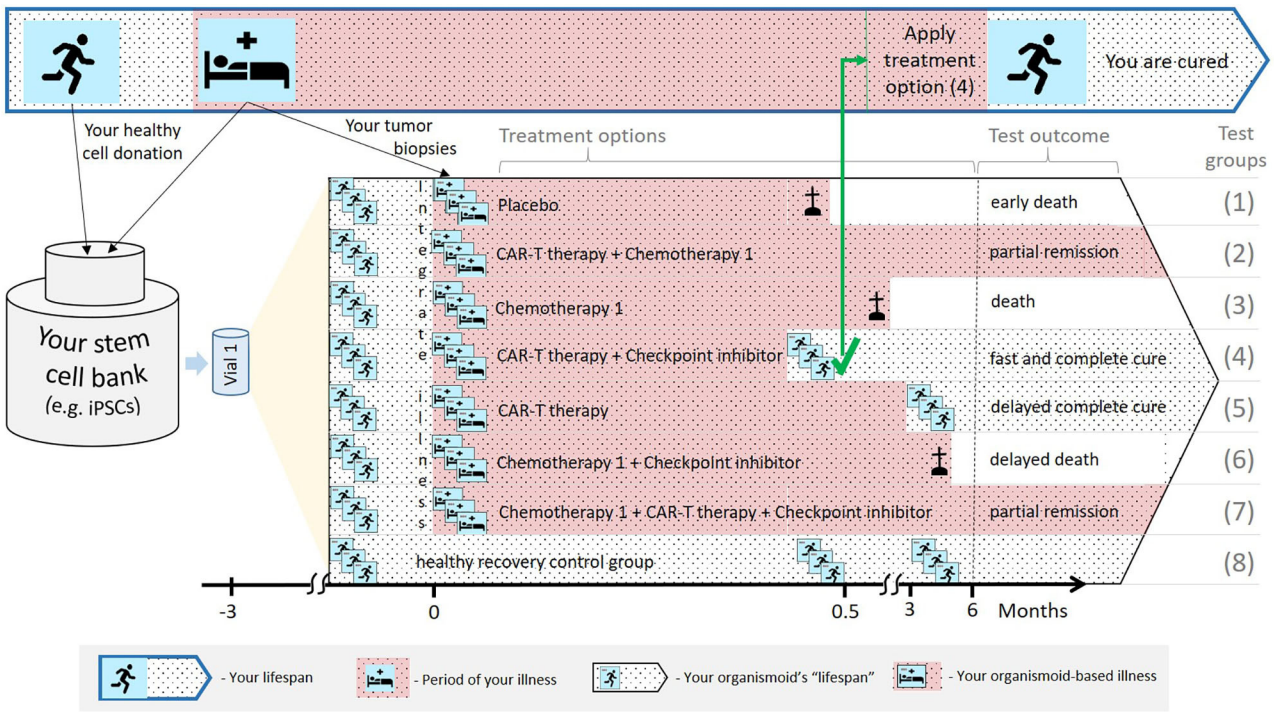

FIGURE 5 | A hypothetical example illustrating how the organismoid theory can be utilized in personalized medicine.

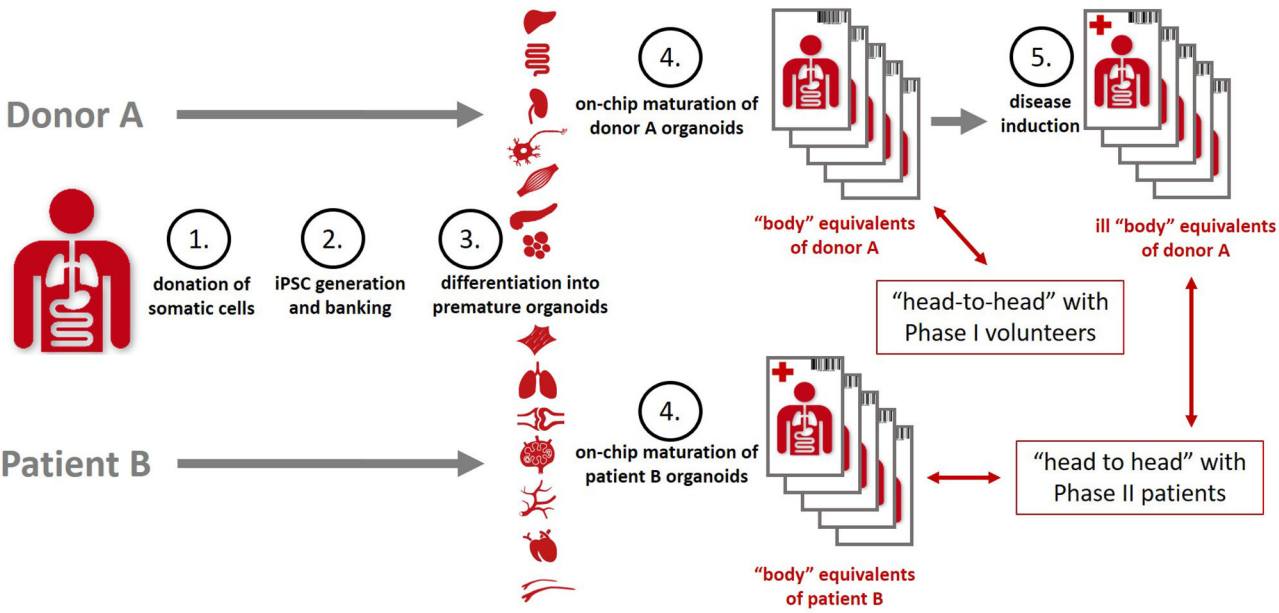

FIGURE 6 | Organismoids—"body" equivalents—in the context of potential "clinical trials" on chips [adopted from (10)].

1 and 2 clinical trials (106). An advanced roadmap toward the qualification of the precision of prediction of "clinical trials" (**Figure 6**) running minute personalized "body" equivalents (organismoids) in on-chip studies head-to-head with clinical trials was sketched in 2018 by the Investigative Toxicology Leaders Forum (10).

In order to achieve that, a set of healthy and diseased organismoids representing the patient's disease status and their

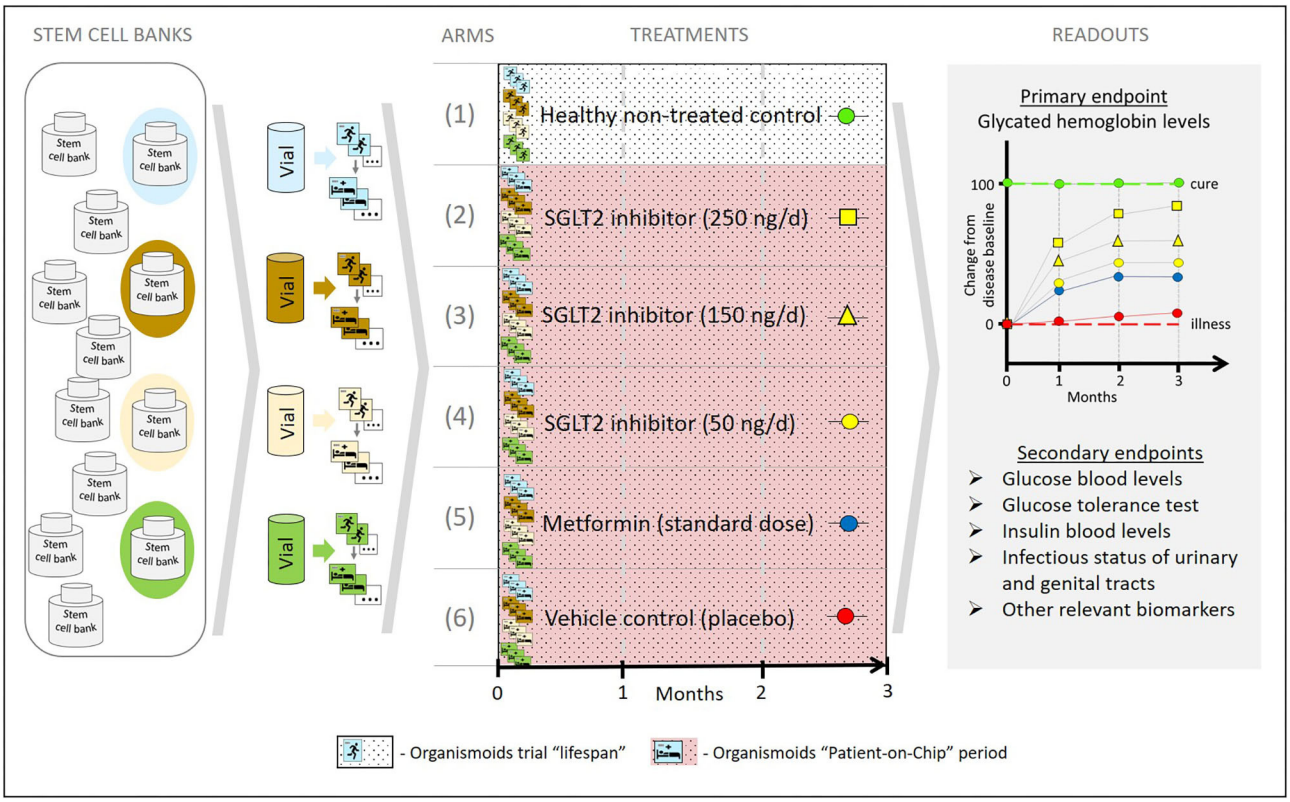

FIGURE 7 | A hypothetical example illustrating how the organismoid theory can be utilized to emulate clinical trials.

healthy homeostasis would allow one to conduct organismoid-based preclinical serial testing of medicines and advanced therapies in a setting emulating clinical trials with large trial-specific patient cohorts. In contrast to trials with patient cohorts, organismoid-based trials offer a number of crucial advantages. **Figure 7** details these advantages by illustrating a hypothetical example of emulating a clinical trial of a hypothetical new sodium glucose transporter 2 (SGLT2) inhibitor to treat diabetes Type 2 utilizing an organismoid-based trial.

Stem cell samples from four donors representing a relevant diabetes patient cohort are collected from a global stem cell bank pool according to criteria equivalent to those of a clinical trial recruitment. A relevant number of healthy organismoids are produced from each of these four donor vials. Blue, brown, yellow and green colors indicate each individual donor background. Subsequently, following the principles of the organismoid theory, diabetic organismoids are generated, for example, through a high glucose diet. In order to evaluate a new SGLT2 inhibitor treatment, a 3 months' trial comprising six arms is conducted with 12 organismoids per arm (three biological repeats per arm and donor) and a daily oral-like administration of the respective medication. The control arms include the healthy recovery group (1) and the diseased untreated placebo group (2) which provide the readout for cure (green baseline) and for no change of disease status (red baseline), respectively. Furthermore, three doses of the new SGLT2 inhibitor are administered at 100,000-fold lower doses than expected in patients, due to the respective downscale of organismoid size in comparison to a human body. Readouts for arms (3), (4), and (5) illustrate the potential of the new monotherapy to change the disease status from baseline toward cure at doses corresponding to 25 mg (yellow squares), 15 mg (yellow triangles), and 5 mg (yellow circles), respectively, per day and patient. A standard monotherapy arm hypothetically treated with metformin (blue circles) provides current standard care reference data. Arm (2) generates the most favorable treatment results in this hypothetical example.

The most prominent advantage is the fact that, for the first time in the history of drug development, organismoid-based trials on chips will include statistically relevant human autologous biological repeats of the patient's body and of the same individual's healthy body status. Due to the lack of any biological repeat for an individual patient and knowledge of their individual biological status at healthy homeostasis, clinical trials traditionally require large cohorts of patients. Therefore, the trials are divided into Phase 1, 2, and 3 and, unfortunately, can only approximate the pathobiology of an individual patient and their complete cure recovery status. Both aspects make the traditional clinical trial process a lengthy and incredibly costly and inefficient way of developing drugs and advanced therapies. "Clinical trials" on chips with healthy and diseased organismoids eliminate these two roadblocks. On the one hand, they allow the uniformity of inbred laboratory animal tests to be matched due to the genetic

identity of each trial "participant" on an individual's organismoid level but with an entirely human background. On the other hand, the usage of organismoids of a variety of different individuals reflects the heterogeneity of patient cohorts in a clinical trial but with the advantage of statistically relevant biological repeats for each individual patient's organismoids.

Another obvious advantage of the organismoid approach is the independence from patient recruitment and hospital usage to conduct such trials. Given the existence of large PSC banks reflecting the genetic predisposition, gender and other categories relevant for the trials, an organismoid-based trial can be conducted at any time, anywhere in the world. Regarding the hypothetical example above, donor selection by diabetic predisposition, comparison of genetic ancestry and equal gender distribution might be interesting stem cell vial selection strategies.

The third advantage to mention is the flexibility regarding the trial size. The number of diseased organismoids (commonly referred to as "patients" on chips) which can be generated is theoretically unlimited. This allows the integration of pharmacokinetic aspects, finding of effective doses and combined safety and efficacy evaluation of a new chemical or biological entity in one and the same organismoid-based trial. Data which are currently generated in separate preclinical and clinical trials in laboratory animals, healthy volunteers and patients, such as the toxicity profile, the no-observed-adverse-effect-level, absorption and excretions rates, metabolite formation, the finding of effective doses, duration and scheduling of a new medication, can result from one organismoid-based trial. Our hypothetical case study to treat Type 2 diabetes could, for example, be easily extended to a larger dose range and a comparison of a single oral-like (this refers in organismoids to any administration to the apical intestine) daily administration with two doses a day. This would include a dose-dependent evaluation of efficacy while simultaneously observing the occurrence and severity of urinal or genital tract infections, well-known side effects of SGLT2 inhibitors. The definition of a therapeutic window for the use of the drug candidate in a respective patient cohort results from such an all-in-one trial, still at a preclinical candidate development stage.

Regarding both usage scenarios, we envisage that organismoids will contribute significantly to medical real-world big data collection from an individual's databases. This is due to the ability to generate unique reproducible data on microenvironmental disruption at the defined location of a first disease hit (e.g., tumor growth, virus replication) for each patient. The combination of organismoid and *in silico* approaches will further increase the predictive power for precise medications for large patient cohorts and reduce costs further.

Sophisticated *in vitro* cell culture work is usually connected in people's minds with high costs. One might speculate that the generation and processing of thousands of organismoids in a trial involves an astronomical budget because currently available MPSs are expensive both in disposable chips and operations. Here, the nature of organismoids—reflecting a self-sustainable human body—and economy of scale effects come into play. In the real world, a human body at rest can be sustained with a daily supply of about 2,000 kcal in proteins, carbohydrates and fat. This can be achieved in some poorer areas of the world for a single digit US dollar bill per person. Consequently, the daily feeding of 100,000 organismoids could be achieved for the same costs. The price of the consumable chips hosting the organismoids will predictively go down to the single dollar range as well, a downscale factor which has already been experienced with computer chips and human genome sequencing costs.

The socioeconomical dimension of the ability of organismoids to identify the best fitting medication for every individual patient and to radically cut costs and transform drug development is envisioned to be enormous. The same applies to the ethical dimension. Human MPS-based organismoids bear the potential to replace the majority of laboratory animal tests and Phase 1 and 2 clinical trials in human volunteers. They will reduce the number of Phase 3 clinical trial patients manifold. All of this will have a radically positive impact on both the patient's benefit and animal welfare on a global scale.

PATIENT'S ORGANISMOIDS AND PATIENT-SPECIFIC T-CELL THERAPIES ON CHIPS—A PERFECT ALLIANCE TO CHALLENGE THE THEORY

Advanced cell therapies, such as the autologous chimeric antigen receptor (CAR) T cell therapies Kymriah™ and Yescarta™, have recently proven their potential to cure former treatment-resistant tumor patients (176, 177). In addition to these two CAR-T cell products approved in 2017 against hematologic tumors, several other CAR-T cell products have recently been approved. Numerous new cell therapy approaches are in the pipeline with CAR or transgenic T-cell receptors against a wide variety of tumors, infections and autoaggressive immune cells, or the use of regulatory T cells to restore immune balance in dominant undesired immune reactions (178). More than 1,000 clinical trials with immune cell products were registered worldwide at the end of 2020 (179).

This unprecedented efficacy in such areas of unmet medical need has spurred regulatory acceptance at the cost of standard safety testing procedures (180), which need to be generated retrospectively in follow-up studies of patients treated after therapy approval. That complies with the fact that a patient's response to a personalized cell therapy cannot be emulated pre-clinically in laboratory animal models because of their phylogenetic distance from the patient, the respective genotypic differences and the immunological mismatch. Similarly, a patient's responses cannot be predicted in conventional patient-derived organoid cultures, due to the lack of their integration into a systemic organismal arrangement. *Inter alia*, the emulation of the intravenous delivery of the T-cell infusion to the target site and its interaction with other major organ sites are missing crucial factors to emulate T-cell therapies and their efficacy

profile in patient-derived organoids precisely. As outlined earlier, the organismoid theory here provides an alternative solution overcoming any remaining obstacles.

WHAT ORGANISMOIDS CANNOT AND SHOULD NOT DO

According to organismoid theory, an organismoid cannot and should not emulate the empathy or consciousness (soul or mind, respectively) which are the major parts of the sociogenesis of a human individual. Consequently, it is not able to model a patient's psychiatric disorders. The dysfunction of a 300 g human heart muscle or hip fracture and its healing rely on biophysical properties, some of which cannot be represented on organismoids due to the mismatch of scale and the physics involved.

Ethical considerations are paramount for human society and are the basis for humanity. Organismoid theory, due to its nature, introduces a number of points which must be considered ethically. Development of the human embryo until a few centimeters in scale is one of the most crucial issues. Fertilization of a human egg and its subsequent embryonic development in an artificial environment (e.g., *in vitro* culture) is prohibited in many parts of the world. The authors of the organismoid theory would like to emphasize that their ethical paradigms extend beyond this. One should not use the concepts and principles of the organismoid theory to create a human or hybrid embryo and further develop and differentiate human or hybrid tissue from that. Other methods should be used to circumvent this part of ontogenesis. The individual's consent to donate tissue to create organismoids could be a good tool to prevent misuse in the areas mentioned at an early stage.

CONCLUSION

The organismoid theory presented here claims the ability to artificially recapitulate the ontogenesis of an individual's body *in vitro*, starting with a donor's stem cells and generating a defined number of identical healthy mature miniaturized body equivalents, termed organismoids, thereof. The theory further claims that such sets of donor-specific identical organismoids reflect a certain stage of that individual's healthy adulthood and can be used to simulate phases of disease and recovery relevant to that donor at a certain time in their lifespan. Modeling the individual's disease in a personalized diseased organismoid approach will provide a yet unmet realistic level of the patient's pathobiology and, consequently, provide an unprecedented tool for selecting precisely the right medicine, therapy schedule and dosing to cure the (diseased) individual.

Nature's principles of genetically and microenvironmentally encoded self-organization and maintenance of the smallest functional units of human organs and their integration into a cross talking and efficiently interacting system of blood perfusion and innervated organs are the blueprint for creating organismoids on chips. We envision them becoming the next level of emulation of human biology, providing the best possible approximation of the human counterpart. Organismoids will organically follow the organoid level of human biology *in vitro*, which, in recent years, has proven to enable the emulation of distinct functions of single tissues and organs at a miniaturized scale. Leveraging on what has been learnt from organoids, human organismoids will add the systemic innervation and supply of whole blood generated on-chip via a miniaturized physiology-based vascular and blood capillary network to the functional units of each organ equivalent. The local separation of the organotypic microenvironments of each organ equivalent from the common bloodstream by the endothelial cell layer will enable the separate organ-specific, genetically encoded and microenvironment-driven self-assembly of exact copies of the functional units of the different human organs on-chip. That, in turn, will enable the physiological cross talk of mature organ equivalents, leading to organismal on-chip homeostasis. Once established, organismoids will only require daily feeding with equivalents of digested food to emulate long-term, so-called self-sustained, body functionality on a chip.

We have illustrated that human organoid *in vitro* culture technologies and human single-organ chips produced within the last 10 years have provided vast evidence for the concept of artificial *in vitro* ontogenesis of single organ equivalents. Furthermore, human iPSC-derived multi-organ chips have furnished first indications of an accelerated artificial organ ontogenesis on chips. Finally, an ever-growing scientific literature on human disease modeling and treatment testing on human tissue chips points toward the capability of such microphysiological platforms to precisely emulate the pathobiology of a disease and the mode of action of a medicine or therapy when organismoids can be fully functionally established on MPSs. Major challenges for the further development of organ-on-a-chip systems are nervous innervation and the implementation of capillarization of the organoids, which also allows the migration of cells, especially immune cells, into the tissue.

We have enrolled the two concepts underlying the organismoid theory and detailed the principles of how to generate and use organismoids for personalized precision medicine.

The prime socioeconomic driver for challenging the organismoid theory is an urgently needed paradigm shift in advanced therapy and drug development for the much faster implementation of affordable advanced therapies and precision medicines into real-world healthcare to cure patients with unmet medical needs. The prime ethical driver is the replacement of the majority of laboratory animal tests and Phase 1 clinical trials on healthy volunteers in the drug development cycle and the shift from treating symptoms toward a curing paradigm for chronic diseases on a global healthcare level. Therefore, we have proposed accelerating the establishment of human

organismoids by their first proof of concept studies in predicting the outcome, dosing and scheduling of advanced autologous T-cell therapies.

OUTLOOK

The MPS community envisions the first proof-of-theory for organismoids to occur within a decade, tackling the radical improvements for the healthcare system described. However, major milestones, such as the interconnection of organ equivalents by a biological vasculature and lymphatics, organ innervation, integration of microbiota, an enterohepatic circulation and, finally, a human-relevant degree of hematopoiesis need to be achieved to accomplish this aim. Above and beyond healthcare, human organismoids, once established, will provide a unique tool for the next level of basic discoveries in the life science of humans. The stable long-term functionality on such a tiny scale and the arbitrary variability in gender, age and genetic background of individual body organismoids will enable previously unimagined insights into human biology. We foresee the use of organismoids for predicting optimized diets, including the adaptation of the microbiome, on an individual or subpopulation level. The development of optimized functional synthetic food to feed the global population beyond 2,100 can be effectively guided by organismoids. The latter will serve as sensitive personalized biological sensors for environmental pollution in air and drinking water and identify potential hypersensitivity risks for their donors. Finally, they bear the potential to become the prime tool for the personalized prediction of measures to ensure the longevity of their respective donors.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6745596/.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, drafted the manuscript, revised it and approved it for publication. UM and RL developed the concept. RD, LK, and AW performed, analyzed and interpreted experiments relating to **Figure 3**. LK, APR, and E-MD performed, analyzed and interpreted experiments relating to **Figure 4**.

FUNDING

Part of this work was supported by the German Federal Ministry of Education and Research (GO-Bio 3B grant agreement No. 031B0062 and Alternativmethoden – Verbund grant agreement No. 031L0099A) and the European Union's Horizon 2020 research and innovation program (RESTORE grant agreement No. 820292).

ACKNOWLEDGMENTS

We wish to thank Philip Saunders for orthographical support with the manuscript. We thank Conor Parks, Surya Kotha, Kainat Khan, and Gareth Maglennon for the generation of cells and their identification in **Figure 3**. We thank Nora Huck for helping with **Figure 5**.

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Conflict of Interest: UM is a founder, shareholder, and the CSO of TissUse GmbH, an organ-on-a-chip company dealing with MPS-based biological models. RL is a shareholder of TissUse GmbH. APR, AW, EA, HE, LK, and E-MD are employed by TissUse.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Medicine

REVIEW
published: 20 September 2021
doi: 10.3389/fmed.2021.728496

When Origin Matters: Properties of Mesenchymal Stromal Cells From Different Sources for Clinical Translation in Kidney Disease

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Advanced therapy medicinal products (ATMPs) offer new prospects to improve the treatment of conditions with unmet medical needs. Kidney diseases are a current major health concern with an increasing global prevalence. Chronic renal failure appears after many years of impairment, which opens a temporary window to apply novel therapeutic approaches to delay or halt disease progression. The immunomodulatory, anti-inflammatory, and pro-regenerative properties of mesenchymal stromal cells (MSCs) have sparked interest for their use in cell-based regenerative therapies. Currently, several early-phase clinical trials have been completed and many are ongoing to explore MSC safety and efficacy in a wide range of nephropathies. However, one of the current roadblocks to the clinical translation of MSC therapies relates to the lack of standardization and harmonization of MSC manufacturing protocols, which currently hinders inter-study comparability. Studies have shown that cell culture processing variables can have significant effects on MSC phenotype and functionality, and these are highly variable across laboratories. In addition, heterogeneity within MSC populations is another obstacle. Furthermore, MSCs may be isolated from several sources which adds another variable to the comparative assessment of outcomes. There is now a growing body of literature highlighting unique and distinctive properties of MSCs according to the tissue origin, and that characteristics such as donor, age, sex and underlying medical conditions may alter the therapeutic effect of MSCs. These variables must be taken into consideration when developing a cell therapy product. Having an optimal scale-up strategy for MSC manufacturing is critical for ensuring product quality while minimizing costs and time of production, as well as avoiding potential risks. Ideally, optimal scale-up strategies must be carefully considered and identified during the early stages of development, as making changes later in the bioprocess workflow will require re-optimization and validation, which may have a significant long-term impact on the cost of the therapy. This article provides a summary of important cell culture processing variables to consider in the scale-up of MSC manufacturing as well as giving a comprehensive review of tissue of origin-specific biological characteristics of MSCs and their use in current clinical trials in a range of renal pathologies.

Keywords: mesenchymal stromal cells (MSCs), tissue source, good manufacturing practice (GMP), advanced therapy medicinal products (ATMPs), kidney disease, cell therapy, clinical application

OPEN ACCESS

Edited by:
Hans-Dieter Volk, Charité Medical University of Berlin, Germany

Reviewed by:
Guido Moll, Charité – Universitätsmedizin Berlin, Germany
Zhengzhao Liu, Central South University, China

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *21 June 2021*
Accepted: *19 August 2021*
Published: *20 September 2021*

Citation:
Calcat-i-Cervera S, Sanz-Nogués C and O'Brien T (2021) When Origin Matters: Properties of Mesenchymal Stromal Cells From Different Sources for Clinical Translation in Kidney Disease. Front. Med. 8:728496. doi: 10.3389/fmed.2021.728496

INTRODUCTION

According to the World Health Organization 2019 Global Health Estimates, chronic diseases are one of the leading causes of mortality worldwide (1). Amongst them, chronic kidney disease accounts for 11–13% global prevalence (2, 3). Based on the course of the injury, kidney diseases and their spectrum of clinical manifestations are stratified into acute kidney injury (AKI), chronic kidney disease (CKD) and end-stage renal disease (ESRD) (4, 5). Persistent loss of kidney function over time leads to kidney failure and at that stage, the current standard of care includes renal-replacement therapies (RRT) (mainly hemodialysis and peritoneal dialysis), or organ replacement. Both strategies suffer significant drawbacks that underpin the need for new preventive and therapeutic approaches.

Cell-based regenerative therapies have the potential to change the paradigm of conventional clinical care. The use of complex biological entities such as cells to promote tissue regeneration and homeostasis, provides a therapeutic alternative to treat and even cure a wide range of diseases. The current cell-based clinical landscape in kidney disease uses hematopoietic stem cells (HSCs), mesenchymal stromal cells (MSCs), and a wide range of blood-derived cells, such as T cells, natural killer (NK) cells, and dendritic cells (6, 7). Notably, blood cell-based therapies using myeloid and T cells are gaining relevance as cellular immunotherapy products to regulate the immune response after procedures such as kidney transplantation (8, 9).

On the other hand, MSCs, which are considered an advanced therapy medicinal product (ATMPs) under EU regulation, have been extensively investigated during the last decade due to their ability to inhibit inflammation and initiate tissue regeneration. The immunomodulatory and anti-inflammatory effects, *via* interactions with immune cells, together with paracrine secretions of anti-apoptotic, anti-fibrotic and matrix remodeling factors, are the main MSC-mediated mechanisms contributing to kidney protection and regeneration (10–12) (**Figure 1**). The effectiveness of MSCs in the treatment of a variety of nephropathies has been largely investigated in pre-clinical models, showing promising results (13). This has encouraged the translation of their use in clinical settings and currently, several early-phase clinical trials have been completed, and many are ongoing, to explore MSC safety and efficacy in renal transplantation, autoimmune diseases, and organ regeneration, especially in late-stage chronic kidney disease patients (**Table 1**). Nevertheless, the road to their routine use in the clinic is far from being a reality. Results in the clinical arena have highlighted the need for better defined therapeutic products. The intrinsic heterogeneity of MSCs in addition to efficacy and safety needs to be extensively investigated before they become a sustainable and affordable therapy (43–45).

One of the current roadblocks relates to a lack of standardization of manufacturing protocols across laboratories and manufacturing centers, which hinders inter-study comparisons within the field (46) and may have significant effects on cell phenotype and performance (47–49). Heterogeneity within MSC populations is another major obstacle; there is now a growing body of literature highlighting unique and intrinsic properties according to tissue origin and donor-related features, with characteristics such as sex, age and disease status having shown to affect their properties (50–54). In this regard, although clinical data has provided evidence for the safety of MSCs (55), attention has also been given to the immune compatibility and hemocompatibility of specific MSC infusions, urging the inclusion of HLA mismatch assessment and expression of procoagulant factors within the safety release criteria (44, 46, 56).

MSCs were initially discovered by Friedenestein et al. as a non-hematopoietic population of cells within the bone marrow (BM), that were plastic-adherent, had fibroblastic phenotype, were able to generate colonies *in vitro* and undergo osteogenic differentiation (57, 58). Later, several groups identified their ability to differentiate into other mesodermal lineages such as adipocytes and chondrocytes, and their ability to be sub-passaged and expanded *in vitro* (59, 60). Since then, MSCs have undergone an extensive diversification and cells with similar characteristics have been isolated from nearly every vascularized tissue (61) as a subgroup of pericytes that reside near vessels, contributing to their homeostasis and regenerative processes (62–64). As a summary, MSCs have been obtained from adult tissues such as adipose tissue (AT-MSCs) (65), dental pulp (DP-MSCs) (66) and other dental tissues (67), endometrium (EM-MSCs) (68, 69), menstrual blood (Men-MSCs) (70), peripheral blood (PB-MSCs) (71, 72) and from several perinatal and birth-associated tissues, referred hereafter as perinatal tissue-MSCs (PT-MSCs) including MSCs from amnion membrane (AM-MSCs), amniotic fluid (AF-MSCs), umbilical cord blood (CB-MSCs), placenta (PL-MSCs), umbilical cord tissue (UC-MSCs) and Wharton's jelly (WJ-MSCs) (73–78) (**Figure 2**). It is important to note that placental tissue can be fetal or maternal in origin, and therefore, MSCs derived from the two types of tissue should be individually characterized.

Current studies focus on trying to understand the mechanistic characteristics underlying MSC-like cells and their therapeutic effects with respect to the tissue of origin. To date, little is known about tissue-specific properties being able to predict clinical efficacy. Considering the significant effect that origin may have on functional properties, and possible therapeutic outcomes, it has now been recognized that the choice of cell source should be considered when optimizing manufacturing protocols for particular clinical applications. In addition to this attention to the source of MSCs, efforts should focus on developing more homogeneous manufacturing approaches to reduce inter-study variability and improve the interpretation and comparability of results from different centers, which ultimately will help to advance the field. Nevertheless, it seems plausible that an ultimate consensus or harmonization will not be reached due to reasons such as intellectual property as well as infrastructure and resources available for large-scale production.

In this article we provide a comprehensive review on the origin-specific biological characteristics of MSCs and their use in current clinical trials in a range of renal pathologies, and attempt to identify intrinsic biological characteristics with beneficial effects. We have also reviewed the literature regarding culture

TABLE 1 | Summary of clinical trials in KD using MSC registered at ClinicalTrials.Gov. Search done on 23rd April 2021.

NCT number	Status	Phases	Start date	Cell source	Donor source	Dose frequency (N)	Infusion route	Results	References
Acute Kidney Injury									
NCT00733876	Completed	Phase 1	2008	BM-MSC	Allogeneic	2×10^6 cells/kg body mass $N = 1$	Ia	No AE or SAE ↓40% hospitalization stay and readmission CKD was stable up to 16 mo follow-up No hemodialysis required	(14–17)
NCT01602328	Completed	Phase 2	2012	BM-MSC[a]	Allogeneic	2×10^6 cells/kg body mass $N = 1$	Iv	~Recovery, need for dialysis, 30-day mortality, AE and SAE between treated and control groups	(18)
NCT01275612	Withdrawn	Phase 1	2010	BM-MSC	Autologous	1 × 10^6 cells/kg $N = 1$	Iv	Patients evaluated not meet the primary criterion	(19)
NCT04194671	Not yet recruiting	Phase 1|Phase 2	2020	UC-MSC	Allogeneic	NA $N = 2$, 7 d apart	Iv		
NCT03015623	Active, not recruiting	Phase 1|Phase 2	2017	BM-MSC[2]	Allogeneic	SBI-101 + 2.5×10^8 vs. 7.5×10^8	Time of hemodialysis		
NCT04445220	Recruiting	Phase 1|Phase 2	2020	BM-MSC[b]	Allogeneic	SBI-101 + 2.5×10^8 vs. 7.5×10^8	Time of hemodialysis		
Sepsis-Induced AKI									
NCT02421484	Completed	Phase 1	2015	BM-MSC	Allogeneic	0.3 vs. 1 vs. 3×10^6 cells/kg body mass $N = 1$	Iv	No AE or SAE ~Efficacy between treated and control groups	(20)
NCT03369275	Not yet recruiting	Phase 2	2018	BM-MSC	Allogeneic	3×10^6 cells/kg body mass $N = 1$	Iv		
Chronic and End-Stage Kidney Disease									
NCT02966717	Active, not recruiting	Phase 2	2016	BM-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 2$, 2 weeks apart	iv		
NCT02166489	Completed	Phase 1	2014	BM-MSC	Autologous	2×10^6 cells/kg body mass $N = 1$	iv	No AE or SAE ~ Renal function	(21)
NCT02195323	Completed	Phase 1	2014	BM-MSC	Autologous	2×10^6 cells/kg body mass $N = 1$	iv		
NCT03321942	Unknown status	Phase 1	2017	AT-MSC	Autologous	NA	iv		
NCT03939741	Recruiting	Phase 1|Phase 2	2019	SVF	Autologous	1×10^6 in 5 mL $N = 1$	iv		

(Continued)

TABLE 1 | Continued

NCT number	Status	Phases	Start date	Cell source	Donor source	Dose frequency (N)	Infusion route	Results	References
Focal Segmental Glomerulosclerosis									
NCT02382874	Completed	Phase 1	2015	BM-MSC	Autologous	2×10^6 cells/kg body mass $N = 1$	iv		
Atherosclerotic Renovascular Disease									
NCT04392206	Recruiting	Phase 1	2020	AT-MSC	Allogeneic	3 vs. 5×10^6 cells/kg body mass $N = 1$	Time of hemodialysis		
NCT01840540	Completed	Phase 1	2013	AT-MSC	Autologous	1×10^5 vs. 2.5×10^5 cells/kg body mass $N = 1$	ia	↑ Cortical perfusion ↑ Renal blood flow ↓ Tissue hypoxia ↑ GFR 3 mo follow-up	(22)
NCT02266394	Completed	Phase 1	2014	AT-MSC	Autologous	NA	ia		
Diabetic Nephropathy									
NCT01843387	Completed	Phase 1|Phase 2	2013	BM-MSC[c]	Allogeneic	1.5 vs. 3 10^8 cells $N = 1$	iv	Trend to stabilized or improved eGFR	(23)
NCT03288571	Not yet recruiting	Phase 1|Phase 2	2019	UC-MSC	Allogeneic	NA $N = 3$, 2 w apart in each kidney	intra-renal		
NCT04216849	Recruiting	Phase 1|Phase 2	2020	UC-MSC	Allogeneic	1.5×10^6 cells/kg body mass $N = 5$, course of 32 w	iv		
NCT04562025	Recruiting	Phase 1	2020	UC-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 3$, weekly	iv		
NCT02585622	Recruiting	Phase 1|Phase 2	2017	BM-MSC[d]	Allogeneic	0.8 vs. 1.6 vs. 2.4 $\times 10^8$ cells $N = 1$	iv		
NCT04125329	Recruiting	Phase 1	2020	UC-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 3$, monthly	iv		
NCT03840343	Recruiting	Phase 1	2019	AT-MSC	Autologous	2.5 vs. 5×10^5 cells/kg body mass/ $N = 2$, 3 mo apart	ia		
Lupus Nephritis									
NCT00698191	Completed	Phase 1|Phase 2	2007	BM-MSC/UC-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 1$	iv	↓ Proteinuria ↑ Disease improvement	(24–26)
NCT01741857	Completed	Phase 1|Phase 2	2012	UC-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 2$, 7 d apart	iv	↓ Proteinuria ↑ Disease improvement	(27)
NCT00659217	Unknown status	Phase 1|Phase 2	2008	BM-MSC	Autologous	NA $N = 1$	iv		
NCT01539902	Withdrawn	Phase 2	2012	UC-MSC	Allogeneic	5×10^7 $N = 2$, 7 d apart	iv	∼ Remission rates in treated and placebo groups	(28)
NCT03580291	Not yet recruiting	Phase 2	2018	UC-MSC	Allogeneic	2×10^6 cells/kg body mass $N = 2$, 7 d apart	iv		

(Continued)

TABLE 1 | Continued

NCT number	Status	Phases	Start date	Cell source	Donor source	Dose frequency (N)	Infusion route	Results	References
NCT03458156	Active, not recruiting	Phase 1	2017	UC-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 1$	iv		
NCT03174587	Completed	Phase 1	2017	BM-MSC	Allogeneic	1, 2 and 3×10^6 cells/kg body mass[◁] $N = 3$	iv	No AE Infusion was tolerated	(29)
NCT04522505	Active, not recruiting	Phase 1	2017	BM-MSC	Allogeneic	1, 2, 3 and 10^6 cells/kg body mass[◁] $N = 3$	iv		
NCT04835883	Recruiting	Phase 2	2019	BM-MSC	Allogeneic	2×10^6 cells/kg body mass $N = 2$, 12 d apart	iv		
NCT04318600	Completed	Phase 1	2014	Amniotic-MSC	Allogeneic	1×10^6 cells/kg body mass $N = 3$, monthly	iv		
NCT03917797	Recruiting	Phase 2	2019	UC-MSC	Allogeneic	NA	iv		
NCT03673748	Not yet recruiting	Phase 2	2021	BM-MSC	Allogeneic	1.5×10^6 cells/kg body mass $N = 1$	iv		
NCT02633163	Recruiting	Phase 2	2018	UC-MSC	Allogeneic	1 vs. 5×10^6 cells/kg body mass $N = 1$	iv		
Kidney Transplant									
NCT00659620	Unknown status	Phase 1|Phase 2	2008	BM-MSC	Autologous	NA	iv		
NCT00658073	Completed	Phase 1	2008	BM-MSC	Autologous	1-2 $\times 10^6$ cells/kg body mass $N = 2$, 24 h and 2 w after Tx	iv	↓ Acute Rejection ↓ Risk of opportunistic infections, ↑ eGFR 1-year follow-up	(30)
NCT00734396	Completed	Phase 1|Phase 2	2009	BM-MSC	Autologous	1×10^6 cells/kg body mass $N = 1$	iv	No AE Resolution of tubulitis without IF/TA in two patients	(31)
NCT00752479	Completed	Phase 1|Phase 2	2008	BM-MSC	Allogeneic	2×10^6 cells/kg body mass $N = 1$, 7 d post Tx	iv	↑ Serum Creatinine > Acute Graft Dysfunction ↑ Regulatory T cells ↓ Memory $CD8^+$ T cells	(32)
						2×10^6 cells/kg body mass $N = 1$, Tx	iv	↓ Memory CD8+ T cells ↓ Donor-specific CD8+ T cell cytolytic response ↑ Expansion of $CD4^+CD25^+FoxP^+$ Treg cells	(33)
NCT02012153	Recruiting	Phase 1	2013	BM-MSC	Autologous	2×10^6 cells/kg body mass $N = 1$, 1 d before Tx	iv	↑ Graft function for 5 to 7 years follow-up ↓ $CD8^+$ T cell in 3 of 4 patients ↓ *ex vivo* T cell donor-specific cytotoxicity	(34)

(Continued)

TABLE 1 | Continued

NCT number	Status	Phases	Start date	Cell source	Donor source	Dose frequency (N)	Infusion route	Results	References
								↑ $CD4^+CD25^+FoxP^+$ Treg cells ↑ Naïve and transitional B cells. 1 patient successfully discontinued immunotherapy with CsA	
NCT02492490	Unknown status	Phase 1|Phase 2	2014	SVF	Autologous	1×10^6 cells/kg body mass *N* = 4, 0, 7, 14, 21 d after Tx	iv		
NCT02561767	Completed	Phase 1|Phase 2	2015	BM-MSC	Autologous	1×10^6 cells/kg body mass *N* = 4, 0, 7, 14, 21 d after Tx	iv	No AE or SAE ↑ GFR. Renal function stable ↑ B cell levels	(35)
NCT02563366	Unknown status	Phase 1|Phase 2	2015	BM-MSC	Allogeneic	1×10^6 cells/kg body mass *N* = 4, 0, 7, 14, 21 d after Tx	iv		
NCT02490020	Completed	Phase 1	2016	UC-MSC	Allogeneic	iv: 2×10^6 cells/kg body mass, 48 h before Tx +/− ia: 5×10^6 cells/kg body mass, during Tx *N* = 2	iv + ia	No AE No MSC engraftment → Post-operative complications → eGFR	(36, 37)
NCT02563340	Unknown status	Phase 1|Phase 2	2015	BM-MSC	Allogeneic	1×10^6 cells/kg body *N* = 4, 2 w apart	iv		
NCT02492308	Unknown status	Phase 1|Phase 2	2014	SVF	Autologous	1×10^6 cells/kg body *N* = 4, 0, 7, 14, 21 d after Tx	iv		
NCT02409940	Completed	Phase 1	2013	BM-MSC	Allogeneic/ Autologous	0.2-3 $\times 10^6$ cells/kg body *N* = 2, 1 d pre- and 30 d post-Tx	iv	No AE or SAE ↑ Graft function ↑ $CD4^+CD25^+FoxP^+$ Treg cells ↓ $CD4^+$T cell proliferation	(38, 39)
NCT02565459	Recruiting	Phase 1	2015	BM-MSC	Allogeneic	1 vs. 2×10^6 cells/kg body mass *N* = 1, Tx	iv		
NCT02387151	Completed	Phase 1	2015	BM-MSC	Allogeneic	1.5 - 2×10^6 cells/kg body mass *N* = 2	iv		(40)
NCT02057965	Active, not recruiting	Phase 2	2014	BM-MSC	Autologous	1 vs. 2×10^6 cells/kg body mass *N* = 2, 6 and 7 w after Tx	iv		(41)
NCT03478215	Recruiting	Phase 2	2016	BM-MSC	Autologous	1, 2, and 3×10^6 cells/kg body mass◁ *N* = 1	iv at surgery		
NCT01429038	Completed	Phase 1|Phase 2	2012	BM-MSC	Allogeneic	1.5 vs. 3×10^6 cells/kg body mass *N* = 2, 3 and 5 d post Tx	iv	No MSC engraftment 2 Kidney/MSC HLA MM 1 MSC MM	(42)

MSC From Commercial Entities: [a]AC607 (AlloCure Inc.), [b]SBI-101 plasmapheresis device in combination with MSC (Sentien Biotechnologies Inc.), [c]Rexlemestrocel-L (Mesoblast Ltd.), [d]ORBCEL-M™ (Orbsen Therapeutics Lt.).
∼, Similar; ↑, Increase; ↓, Decrease; ◁, Dose-escalated study.
AE, Adverse events; AKI, Acute Kidney Injury; AT, Adipose Tissue; BM, Bone Marrow; CsA, Cyclosporin A; d, day; eGFR, estimated Glomerular Filtration Rate; GFR, Glomerular Filtration Rate; HLA, Human Leukocyte Antigen; ia, intra-arterial; IF, Interstitial fibrosis; iv, intra-venous; Kg, Kilogram; MM, Mismatch; mo, month; MSC, Mesenchymal Stromal Cell; NA, Not Available; SAE, Severe adverse events; SVF, Stromal Vascular Fraction; TA, Tubular Atrophy; Treg, Regulatory T cells; Tx, Transplant; UC, Umbilical Cord; w, week; y, year.

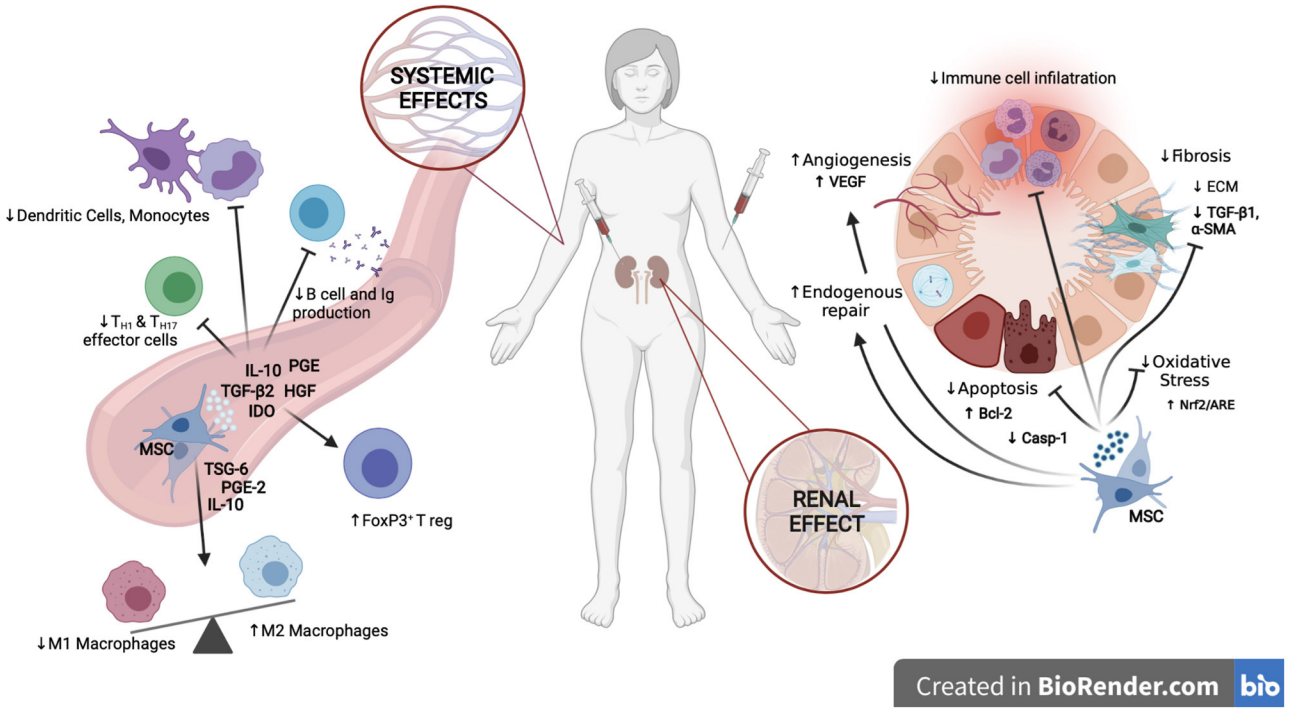

FIGURE 1 | Mechanisms of action of MSCs in kidney disease. At the renal level, MSCs enhance endogenous mechanisms of repair, confer cytoprotection by dampening apoptosis and oxidative stress, promote vascular preservation and regeneration, diminish renal fibrosis and reduce infiltration of immune cells, creating an anti-inflammatory and pro-regenerative environment. At the systemic level, MSCs inhibit the pro-inflammatory activities of both, the innate and adaptative immune system, enhancing the expansion of tolerogenic T reg and M2 Macrophages while inhibiting M1 macrophages, monocytes, dendritic cells, and T and B lymphocytes. Created in BioRender.com.

processing variables that are important to consider during the scale-up and manufacture of the cell product. As part of this study, we conducted a search in the ClinicalTrials.gov database of current registered clinical trials on kidney disease. According to our search, fifty-four clinical trials are being or have been conducted around the world to study the safety and efficacy of MSC-based ATMPs in a variety of renal diseases (accessed in April 2021, https://clinicaltrials.gov) (**Table 1**). We acknowledge that this list may not be exhaustive as it is derived from one well-known clinical trial registry, and it is possible that some other clinical trials may be listed in other national or international registries, which have not been considered in this review. The search includes clinical studies at all different stages (completed, recruiting, or not enrolling). Search terms included: "mesenchymal stem cells," "mesenchymal stromal cells," "kidney injury," "kidney disease," "kidney transplant," combined in various modifications with "AND" and "OR." When possible, information about MSC tissue source, donor (allogeneic or autologous), and cell processing variables such as cell plating densities, passage number, culture media supplements and culture devices for cell expansion, were extracted (**Table 2**). When available, additional sources of information were retrieved from hand searches of relevant papers and/or websites.

MSC-BASED THERAPIES IN KIDNEY DISEASES

Disease Overview

Generally, kidney diseases have been subdivided into acute kidney injury (AKI) and chronic kidney disease (CKD), according to the duration of the injury. While AKI is described as an abrupt decline in renal function, CKD emerges after years of progressive and persistent loss of glomerular filtration rate and albuminuria (4, 5). Although they were originally considered two individual entities, it is now clear they share an intrinsic link: maladaptive repair following AKI leads to progressive CKD, and at the same time patients with underlying CKD are more likely to develop AKI resulting in a deterioration in renal function (82). Often asymptomatic, the progressive nature of CKD leads to a vicious cycle of injury that ultimately causes renal failure or end-stage renal disease (ESRD) (83). At the time of late-stage CKD diagnosis, renal function has declined beyond physiological reserve and kidney failure is diagnosed. Despite significant advances in understanding the pathophysiology of AKI and CKD, current therapeutic and pharmacological approaches only offer supportive treatment to handle and manage underlying complications (84). In recent years MSCs and their derived by-products, mainly paracrine signals and extracellular vesicles

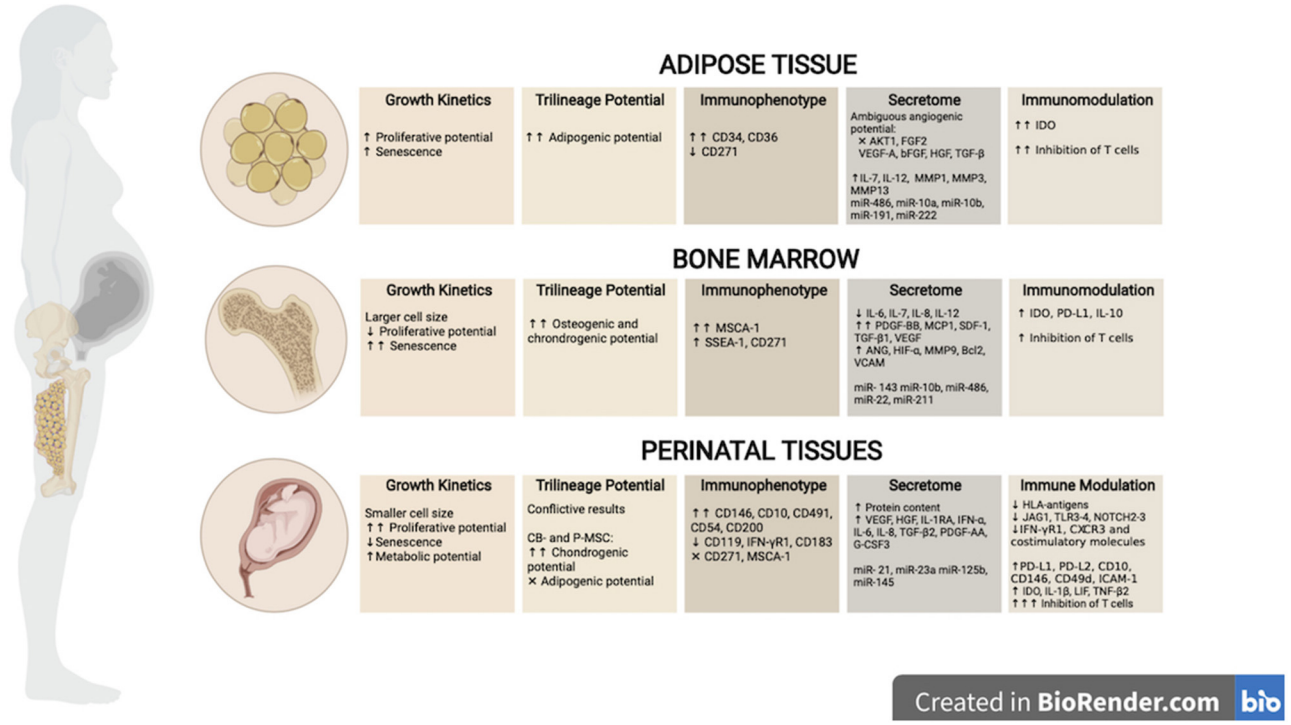

FIGURE 2 | Biological properties of tissue-derived MSCs. MSCs can be isolated from adult tissue sources such as adipose (AT)- and bone marrow (BM), as well as perinatal and/or birth-associated tissues, including amniotic liquid (AM), cord blood (CB), placenta (P) or umbilical cord (UC) tissues. Tissue of origin have shown to impact the biological properties of MSCs. This figure illustrates the main differences described in the literature regarding growth kinetics, differentiation abilities, immunophenotype, secretome, and immune modulation between cell sources. Created in BioRender.com.

(EVs), have emerged as a novel cell-based therapy to treat acute and chronic kidney injury. Herewith, this section reviews the growing body of preclinical and clinical evidence on the potential role of MSCs in recovery after kidney damage.

Mechanisms of Action of MSCs in Kidney Disease

MSCs in Acute Kidney Injury

AKI is considered a severe clinical syndrome in hospitalized patients, with a prevalence of 1–9%, and is especially common among critically ill patients, affecting 45% of patients admitted to the intensive care unit (85). The abrupt decline in renal function is accompanied by an alteration of the homeostasis within the kidney. The decline in glomerular filtration rate results in accumulation of serum creatinine, blood urea nitrogen (BUN), and/or reduction in urine output (85). Along with the original insult, functional disturbances cause a reduction in renal cell mass due to cell death, impairing renal function and facilitating the subsequent progression to fibrosis. Notwithstanding all efforts to manage the associated clinical manifestations, AKI is still considered an independent risk factor for mortality and development of CKD (86). Within the multiple etiologies of renal injury, ischemia-reperfusion injury (IRI) is the most prevalent form of AKI, together with vascular obstructions within the renal circuit (85), and drug-induced nephrotoxicity (87). IRI is also an unavoidable event during kidney transplantation, limiting graft functionality and increasing the risk of rejection and graft loss (88).

Inflammation plays a central role throughout the process of kidney injury (89). Shortly after the injury, activation of inflammatory pathways induces the recruitment and infiltration of leukocytes such as neutrophils, monocytes, and dendritic cells. T and B lymphocytes have also been linked to contributing to kidney injury. Conversely, regulatory T cells and M2 macrophages are essential in suppressing inflammation, enhancing tissue remodeling and causing repair. However, if uncontrolled, the endogenous mechanisms of tissue repair within the kidney could promote additional damage and irreversible fibrosis (90). Together, the immune system, the ischemic environment and the endogenous mechanisms of repair converge in a complex milieu of profibrotic and proinflammatory cytokines and chemokines. In this context, MSCs have been proposed as powerful candidates to dampen the severity of AKI and promote effective regenerative processes.

Infusion of MSCs in several *in vivo* models of AKI has resulted in improved renal function by decreasing tubular injury, promoting angiogenesis, reducing oxidative stress as well as inflammation, promoting a pro-regulatory and anti-inflammatory phenotype (91–93). The main mechanisms whereby MSCs have been found to elicit such renoprotective effects are related to paracrine signaling and shedding of

TABLE 2 | Summary of MSC culture processing variables publicly available from clinical trials of KD.

NCT number	Cell source	Donor origin	Media supplements	Cell densities		Passage number	Cell culture system	References
				Isolation	Plating			
Acute Kidney Injury								
NCT00733876	BM-MSC[a]	Allogeneic					2D Flask	(79)
NCT01602328	BM-MSC[b]	Allogeneic					2D Flask	(18)
NCT03369275	BM-MSC	Allogeneic	XF				3D Bioreactor	
NCT02421484	BM-MSC	Allogeneic	XF				2D Flask	(20)
NCT01840540	AT-MSC	Autologous	5% hPL	100, 000 cells/cm^2	100 – 250 cells/cm^2		2D Flask	(22, 80)
Chronic Kidney Disease								
NCT02166489	BM-MSC	Autologous	10% FCS			P1-3	2D Flask	(21)
Diabetic Nephropathy								
NCT01843387	BM-MSC[b]	Allogeneic	10% FCS			P2	2D Flask	(23)
NCT02585622	BM-MSC[c]	Allogeneic					3D Bioreactor	
Lupus Nephritis								
NCT00698191	BM-, UC-MSC	Allogeneic	10% FCS	100, 000 cells/cm^2	1, 000 cells/cm^2	P2-P5	2D Flask	(24–26)
NCT01741857	UC-MSC	Allogeneic	10% FCS	100, 000 cells/cm^2	1, 000 cells/cm^2	P3-P5	2D Flask	(27)
Kidney Transplant								
NCT01429038	BM-MSC	Allogeneic	10% FCS			P2	2D Flask	(81)
NCT00658073	BM-MSC	Autologous	HSA			P3-4	2D Flask	(30)
NCT00734396	BM-MSC	Autologous	10% FCS	160, 000 cells/cm^2	4, 000 cells/cm^2		2D Flask	(31)
NCT00752479	BM-MSC	Allogeneic	5% hPL	200, 000 cells/cm^2	200 cells/cm^2	12 d in culture	2D Flask	(33)
NCT02492490	SVF	Autologous	10% FCS				2D Flask	
NCT02561767	BM-MSC	Allogeneic	10% FCS				2D Flask	(35)
NCT02490020	UC-MSC	Allogeneic	HSA				2D Flask	(36, 37)
NCT02409940	BM-MSC	Allogeneic/ Autologous	10% hPL	200, 000 cells/cm^2	500, 000 cells/cm^2	P3	2D Flask	(38, 39)
NCT02012153	BM-MSC	Autologous	5% hPL	200, 000 cells/cm^2	100–200 cells/cm^2	P1	2D Flask	(34)

AT, Adipose Tissue; BM, Bone Marrow; FCS, Fetal Calf Serum; HSA, Human Serum Albumin; hPL, Human Platelet Lysate; SVF, Stromal Vascular Fraction; UC, Umbilical Cord; XF, Xenofree media.

MSC Commercial Names: [a]AC607 (AlloCure Inc.), [b]Rexlemestrocel-L (Mesoblast Ltd.), [c]ORBCEL-M^{TM} (Orbsen Therapeutics Lt.).

extracellular vesicles (79, 94). MSC-based therapies have been proven to stimulate the regeneration of tubular epithelial cells by increasing intra-renal levels of HGF (95–97) and TSG6 (98), promoting the activation of pro-survival pathways such as AKT/ERK (99); decreasing tubular apoptosis, by upregulating Bcl2 and downregulating Caspase 3 (100), and inhibiting the endoplasmic reticulum stress response (99). Moreover, MSCs help in counterbalancing the oxidative damage by enhancing the activity of free radical scavengers (101), favoring the activation of the Nrf2/ARE pathway (102) and downregulating the expression of NOX2 which are key ischemia-related insults (102). A large part of the beneficial effect of MSCs is related to their interaction with both, the innate and adaptive immune systems. The complement system serves as a key moderator of the immune system and MSCs have been described to interact with this system in a synergistic manner to modulate the host immune response (103). Conversely, in the context of kidney injury, MSCs have been found to inhibit the overactivation of the complement cascade, decreasing serum levels of C5a as well as intra-renal deposits of C3 and C5aR (104, 105). Downregulation of inflammatory cytokines such as TNFα, MMP9, ICAM1, NFκB (100, 106, 107) and chemokines such as CX3CL1 (108), CXCL2, and IL6, decreased the infiltration of pro-inflammatory macrophages (109) and effector T cells while promoting the presence of regulatory T cells (110). This "shift" toward an anti-inflammatory profile seems to be, in part, governed by the expression of IL10 (111) and adherence factors such as ICAM1 and VCAM1 (112). The secretion of pro-angiogenic factors [e.g., VEGF, eNOS (113–116)] has been shown to improve capillary rarefaction (107), dampening the ischemic damage and preventing the progression of interstitial fibrosis (108, 110).

Interestingly, *in vitro* experiments have found that small single-stranded non-coding RNA molecules (miRs) contained within EVs produced by BM-MSCs can protect proximal tubular epithelial cells after ischemia by targeting the expression of mRNAs associated with apoptosis, cytoskeleton reorganization, fibrosis, and hypoxia (117), endowing EVs and their miR cargos with interactive roles in the regenerative process.

Recently, a novel mechanism of action has been proposed whereby MSCs could rescue damaged tubular cells by targeting mitochondrial dysfunction and sustaining their energy supply (118), and restoring physiological dynamics (119). Another

consideration in the therapeutic use of MSCs is the use of genetic modification (120–122) as well as pre-conditioning strategies such as hypoxic culture conditions (123–125), and priming of cells (126–128), which have showed superior therapeutic potential compared to that of unmodified controls (129).

MSCs in Chronic Kidney Injury and End-Stage Renal Disease

CKD emerges as the result of continuous kidney damage and scarring mediated by a dysfunctional inflammatory status (130, 131). The perpetuation of the injury is often a result of high blood pressure, nephrolithiasis, and several underlying conditions such as diabetes mellitus (10), systemic lupus erythematosis (132), or glomerular pathologies (133), as well as the development of *de novo* AKI (134). Regardless of the initial insult, the exacerbated renal fibrosis response that occurs throughout the course of the disease induces morphological alterations with physiological and functional consequences (135). Progression to ESRD is, therefore, inevitable.

Paracrine signaling and/or EVs derived from MSCs have been transiently found within the glomeruli and injured tubules, limiting the extent of the injury by alleviating interstitial fibrosis, recruiting leukocytes, and activating intrinsic repair mechanisms that prevent AKI-CKD transition (136–139). Similar effects have been described in several models of established CKD, where cell and cell-free strategies resulted in reduced accumulation of fibrotic tissue as a result of decreased expression of extracellular matrix components and increased capillary density, attenuation of the pro-fibrotic and pro-inflammatory environment, and promotion of M2 anti-inflammatory macrophages (140–142). However, attenuation of inflammation is not always achieved, probably due to differences in treatment time and frequency (143). In these circumstances, "licensing" strategies have proven to be efficient in promoting an early onset of MSC therapeutic effects (128).

Several studies have also explored MSC therapies in chronic scenarios where renal damage is being perpetuated by underlying pathologies, predominantly autoimmune nephritis caused by systemic lupus erythematosis (SLE) and microvascular complications of diabetes mellitus, commonly referred to as diabetic nephropathy (DN). In both scenarios, preclinical models have described the usefulness of MSCs in ameliorating the pathogenic manifestations albeit through different mechanisms due to the different nature of the insults. MSCs in preclinical models of lupus nephritis (LN) have been shown to act by suppressing the activation of the humoral and cellular immune response, evoking a systemic pro-tolerogenic milieu (144–146). Besides regulating leukocyte infiltration and inhibiting proinflammatory cytokines, beneficial actions in DN models have been also attributed to the reduction of systemic biochemical alterations and reducing renal levels of oxidative stress, apoptosis, and fibrosis while promoting renal regeneration (147–150).

MSCs in Kidney Transplantation

One of the most relevant clinical settings where MSCs have shown potential beneficial effects is renal transplantation. In murine models of kidney transplantation (KTx), infusion of autologous (151, 152) or syngeneic (153, 154) MSCs induced graft tolerance and recipient survival. The achievement of a pro-tolerogenic environment was, in part, mediated by the production of indoleamine 2, 3 dioxygenase (IDO), crucial in generating regulatory $FoxP3^+$ T cells (112, 151). The effect was increased when BM-MSCs were licensed with the pro-inflammatory cytokine IL17A (152, 155). However, administration of MSCs was found to only elicit a tolerogenic response and enhanced graft survival when administered following graft transplantation (112, 153, 154).

Comparable effects have been reported in rodent models where single (156, 157), and multiple (158) administrations of MSCs resulted in significant improvement in graft function and attenuated expression levels of pro-inflammatory cytokines (156–158). Licensing with TGFβ1 (159) or genetic modifications to overexpress CXCR4 (160) enhanced the immunosuppressive abilities and showed an increased induction of regulatory T cells and anti-inflammatory cytokines. Beneficial effects have also been reported in attenuating cellular infiltration and tubular damage due to chronic graft rejection (156, 161, 162). In contrast with this favorable preclinical evidence, other studies have reported that administration of MSCs and their derived EVs did not exert similar beneficial effects (163, 164), highlighting the impact of timing and synergistic immunosuppressive strategies to ensure robust therapeutic effects.

Clinical Translation of MSC Therapies in Kidney Disease

Promising preclinical results, described above, have led to early-phase clinical studies that investigate the safety and efficacy of MSC-based therapies in a wide range of renal pathologies. Based on data compiled from studies registered on clinicaltrials.gov (accessed in April 2021), a total of 54 been registered since 2008. The main results of our search are summarized in **Figure 3** and expanded in **Table 1**, which present an overview of the clinical use of MSCs in kidney disease highlighting heterogeneity in terms of tissue source and product development characteristics. Results from this search showed that MSCs have been most commonly used to improve outcomes of kidney transplant procedures (31.5%), targeting either induction of allograft tolerance or minimizing the use of immunosuppressive drugs. Other trials have focused on the renoprotective potential of MSCs in lupus nephritis (24%), AKI (14.8%), diabetic nephropathy (13%), and CKD/ESRD (16.7%) (**Figure 3A**). Nevertheless, MSC therapies for these conditions have yet to reach later stage clinical trials and market authorization (**Figure 3B**).

Results from our literature search have also highlighted the great heterogeneity within the field in terms of donor and tissue source, mode of cell delivery and cell dose (**Table 1**). During the last decade, BM-MSCs have been the predominant cell source in clinical trials (7). However, clinical MSC products have greatly diversified in the past decade, with equal use of BM-, AT-, and PT-MSC products in clinical trials (44, 165). In the kidney disease clinical arena, the use of BM-MSCs remains the predominant source (58%), although in the past 10 years a diversification

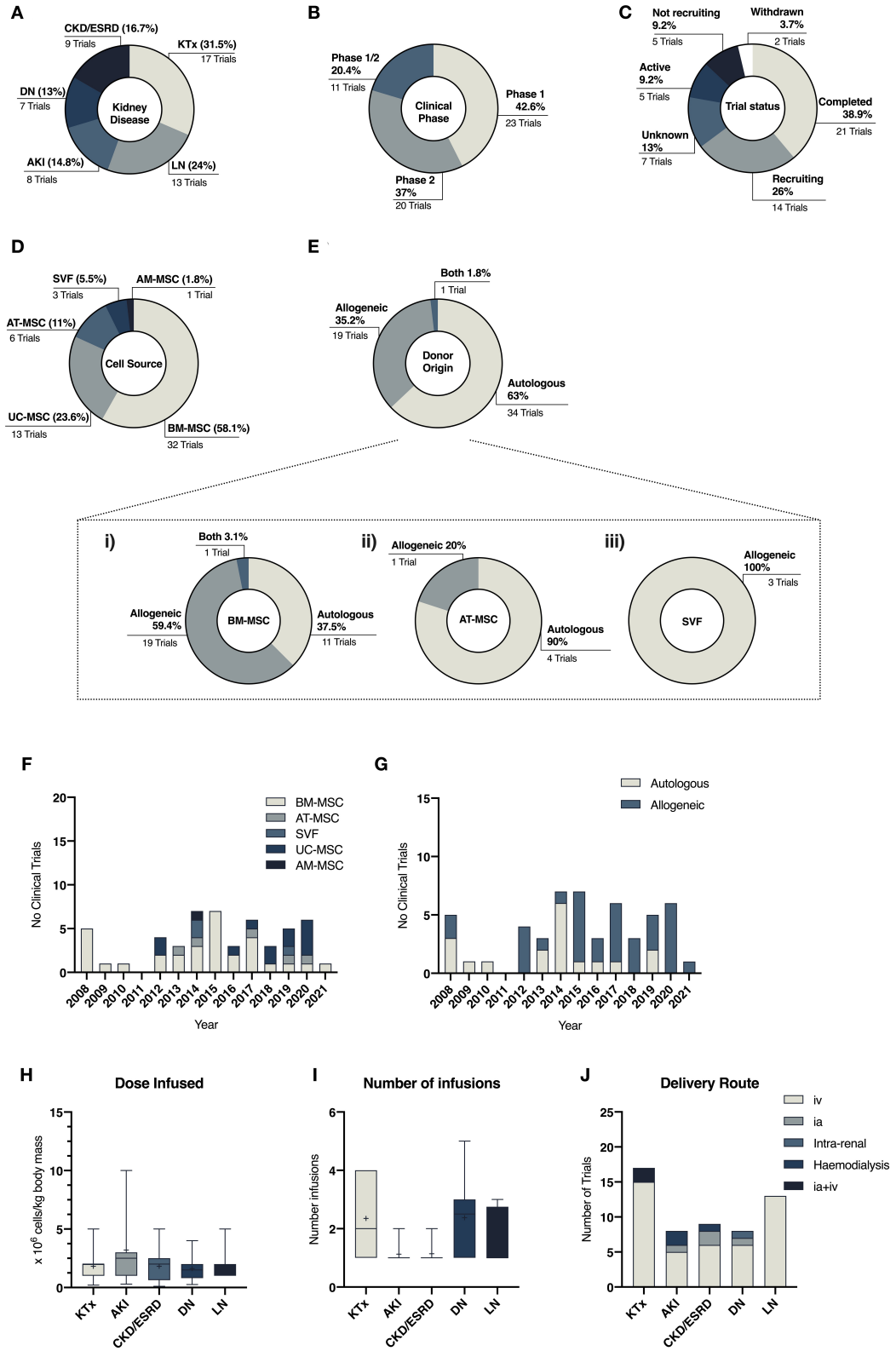

FIGURE 3 | Descriptive data related to clinical trials in kidney diseases comparing the number of trials per disease **(A)**, clinical phase of the studies **(B)** and their status **(C)**. **(D,E)** Illustrate the heterogeneity of cell and donor source across all studies, while **(F,G)** depicts the change of cell and donor source preferences over the years. **(H–J)** illustrate protocol differences across different disease settings related to dose of MSC infused **(H),** frequency of infusions **(I)** and choice of delivery route **(J)**.

on the use of different MSC sources have also been noted (**Figures 3D–G**).

Most trials have used intravenous delivery (**Figure 3J**), despite studies which have shown that the majority of MSCs are trapped within the pulmonary circuit (166, 167). Some studies have explored the combination of intra-arterial delivery, to facilitate homing of the cells within the kidney, and intravenous infusion (35, 36). Nevertheless, in both cases, hemocompatibility and levels of procoagulant tissue factor (TF/CD142) should be considered to avoid the onset of the instant blood-mediated inflammatory reaction (IBMIR), as it is an important aspect for the safety and efficacy of these therapies (168). Although clinical protocols have added anti-thrombotic drugs to ensure the safety of MSC products (169), several studies have reported that TF/CD142 expression varies between MSC tissue origin and is highly impacted by culture processing as well as cell-dosage (44, 168, 170). Given the procoagulant nature of MSCs, safety characterization is of utmost importance and even more so in patients undergoing organ replacement therapies that carry the burden of strong immunosuppressive regimes and their side effects such as increased cardiovascular risk (56, 171).

With the increasing presence of allogeneic therapies in the past years (**Figure 3G**), greater considerations should also be placed on the potential impact of MSC immunogenicity and the generation of alloreactive immune responses, as little is known of its long-term clinical implications (172, 173). Despite the presence of extensive evidence showing anti-donor cellular and humoral immune responses following administration of allogeneic MSCs (174, 175), presence of donor-specific human leukocyte antigen (HLA) antibodies has been minimally considered previously (172). This will represent a major risk when repeated injections are being included in therapeutic procedures for potentially pre-sensitized patients such as those undergoing kidney transplantation (56, 173).

Finally, another aspect to consider is whether MSCs are delivered as culture-adapted or "fresh" cells, with optimal metabolic fitness, or cryobanked "off-the-shelf" cells, which are thawed immediately prior to transplantation. While this is an important aspect for efficacy and safety of MSC therapies, results from our search, and others (176), have reported that the method of cell delivery (fresh vs. cryobanked), is often omitted or not clearly stated in manuscripts. The tendency to use cryobanked "off-the-shelf" cells has increased over time (48, 176), most likely due to the logistic advantages of this approach. However, controversy revolves around the use of banked products, with studies demonstrating reduced therapeutic potential, loss of functionality and increased susceptibility to trigger prothrombotic events (177–181), while others have showed minimal impairment of cell viability and fitness (182, 183). In this context, clinical potency has been linked with the concept of metabolic fitness and product viability at the time of infusion (184, 185) making it critical to develop manufacturing methods to rescue cryopreserved cells and restore cell functionality (186). Intriguingly, other studies have demonstrated that apoptotic or dead MSCs, and therefore less metabolically active, confer therapeutic benefits by enhancing the host innate immune response (187–189). Gaining better mechanistic insights behind the benefits that MSC therapies elicit, may it be due to viable cells, their derived by-products or rather by immune activation through dying/dead cells holds the key to elicit better therapeutic outcomes (186, 190).

Kidney Transplantation

Based on their immune-privileged characteristics, MSCs have been administered in conjunction with RRT to promote graft tolerance and control the host immune system with hopes of enhancing the withdrawal or minimization of immune-suppressive therapies and enhancing organ function. Initial results from a pilot study published by Perico et al. revealed the importance of timing of cell delivery. Autologous infusion of BM-MSCs in two patients seven days after undergoing KTx from a living related donor caused a severe decline in renal function and humoral and cellular acute rejection (32). The post-surgery subclinical inflammatory environment upon which MSCs were transplanted seemed to favor the development of a pro-inflammatory phenotype that could have contributed to an early graft dysfunction (154). Pretransplant administration did not result in impaired graft function, highlighting the paramount relevance of protocol optimization (33). Moreover, it showed a pro-tolerogenic graft environment supported by reduced effector $CD8^+$ T cells and expanded regulatory $CD4^+FoxP3^+$ T cells that led to stable graft function after long-term follow-up (34). In one patient, long-lasting counterbalance of regulatory/effector T cells and increased presence of B cells allowed the successful discontinuation of the use of ciclosporin A and tapering of the dose of immunosuppressive drugs (34).

Several other studies have provided further insights into the applicability of MSC in kidney transplant from living related (31, 35, 36, 38) and deceased donors (36, 191). Using kidneys from brain or cardiac deceased donors would potentially increase the number of transplant recipients and meet the growing need for kidney grafts (192). However, these procedures are associated with a higher incidence of early graft dysfunction and acute rejection as prolonged ischemic time exerts an adverse event on graft survival (193, 194). Recently, the combinatorial infusion of UC-MSCs before and during surgery in recipients of deceased donor grafts was proven to be safe and resulted in no adverse clinical events. However, no significant benefit was seen in terms of reduction of postoperative complications, survival rates and graft function (36, 37). A larger study would possibly facilitate a full assessment of improvement in delayed graft function, as a lower incidence was seen in the MSC treated group (36). In a much smaller trial, third party MSCs were infused in five kidney transplant recipients from deceased donors 3–5 days after the procedure. The 6-month safety interim report revealed no graft rejection but some degree of immunization against the shared kidney and MSC donors (191).

Despite the absence of treatment-related serious adverse events in the studies described so far, a side effect of MSC systemic immunosuppressive activity was reported in a small Phase I study, where three out of six patients developed opportunistic viral infections after MSC-infusion (31). Yet, in a much larger study involving 156 patients, inoculation of BM-MSCs resulted in a significantly decreased risk of opportunistic

infections (30). Although no neoplastia-related events have been described in KTx, this stresses the importance of carefully monitoring MSC preparations and monitoring infused patients, particularly in elderly and chronically immuno-suppressed patients with an increased risk to develop tumors and infections.

To date, a total of eight clinical trials have been completed and results published, while nine more are yet to be completed or with no publicly available results (**Figure 4**). The main differences between studies can be seen between the cell source and dose regimen, as well as infusion timing and frequency (**Figure 4**). So far, BM-MSCs have been the choice of starting material in fourteen studies (82.3%), six using autologous (43.75%) and eight using allogeneic cells (37.5%); only two studies used SVF from autologous fat tissue and one from UC-MSCs (**Figure 4**). On review of the published literature, no conclusions can be drawn to determine differences in clinical outcomes on the tissue source.

Finally, although we have previously discussed the effects of timing, the rationale for administration weeks after surgery seems to be directed toward generating a pro-tolerogenic environment that would help in easing the withdrawal or tapering of immunosuppressive drugs (34). Results from current ongoing studies looking at whether MSCs in combination with mTOR inhibitor everolimus can be used for tacrolimus withdrawal may be able to shed light on the use of MSCs as a long-term effective immune-suppressive strategy (40, 41).

Acute Kidney Injury

Limited attempts with contradictory results have resulted from the exploration of the safety and efficacy of MSCs in recovering renal function after post-cardiac surgery AKI. An exploratory phase I trial studied the safety and feasibility of infusing allogeneic BM-MSCs in patients with several underlying comorbidities at high risk of developing AKI after open-heart surgery (14, 15). Outcomes from the first five patients showed that prevention infusion of MSCs was safe, averted postoperative decline in renal function, and decreased time of hospitalization and rates of readmission. Moreover, patients with underlying CKD had stable renal function and no disease progression after 16 months follow-up (16).

These encouraging results contrast with those from a recently published randomized, double-blind, phase II study with subjects undergoing cardiac surgery with evidence of early AKI development. Administration of commercial allogeneic MSCs (AlloCure Inc.) after AKI development did not improve the time of renal function recovery, rates of adverse events, need for dialysis or 30-day mortality (18). However, the authors recognized that infusion in an overwhelming status of the disease could have hampered the potential benefits. Further studies should aim to determine whether more favorable effects could be seen in prevention studies, such as the trial by Tögel and Westenfelder described above, rather than interventional studies when MSCs are administered after AKI onset (195).

It is well-known that sepsis, among other pathologies, can lead to the development of AKI in critically ill patients (196). A phase I study explored the safety and tolerability of administered allogeneic BM-MSCs in nine patients with septic shock (20). No infusion-associated or serious adverse events were detected, and no AKI outcomes reported. A follow-up phase II study (NCT 03369275) will further examine the efficacy of MSCs in this context.

As the regenerative medicine field evolves, new strategies are being developed to combine the use of cell-based therapies with cutting-edge biomedical devices (197–199). In this context, a phase I study is looking at the safety and tolerability of a biologic/device combination product called SBI-101 (Sentien Biotechnologies, Inc. USA). It combines a plasmapheresis device with allogeneic BM-MSCs and is designed to regulate inflammation and promote tissue repair. Two experimental cohorts using a low and high dose of MSC will be tested in AKI patients receiving continuous renal replacement therapy (NCT03015623). Furthermore, a second phase I/II trial (NCT04445220) aims to explore the use of this same device in COVID-19 patients that develop AKI.

The limited and contradictory clinical data available on the use of MSCs in AKI as well as the lack of mechanistic results challenge the possibility of drawing therapeutic roadmaps to guide the use of stromal cells in this context. Most studies related to AKI have used allogeneic BM-MSCs, emphasizing the relevance of "off-the-shelf" therapies in acute settings, where immediate therapy is needed (**Figure 4**). The exception is a phase I study that aimed to explore the use of autologous BM-MSCs for cisplatin-induced AKI in patients with solid organ cancer (NCT01275612). Unfortunately, none of the screened patients met the primary criterion of acute renal failure, and the study was withdrawn.

In terms of cell product preparations, illustrated in **Figure 4**, limited data is available; intravenous administration or infusion through the left carotid or femoral artery are the preferred delivery option whereas cell doses are inconsistent within studies (**Figure 4**). Timing and frequency of infusion, patient selection, guided by more sensitive biomarkers (200), and cell preparation, are some of the concerns that will have to be addressed in future preclinical and clinical studies to establish reliable therapeutic strategies.

Chronic Kidney Injury and End-Stage Renal Disease

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disease characterized by progressive formation and enlargement of cysts in multiple organs that have a critical effect on kidneys. The infusion of autologous BM-MSCs in a small cohort of ADPKD patients was safe and well-tolerated albeit did not improve renal function (21).

Atherosclerotic renovascular disease is the most common cause of secondary hypertension and leads to deterioration of renal function due to insufficient vascularization and ischemia (201). Current treatments based on blood flow restoration have proven unsuccessful to recover kidney injury upon damage (202). Results from a phase I study showed increased cortical perfusion and decreased renal hypoxia after infusion of autologous AT-MSC, suggesting a beneficial effect of MSCs through amelioration of the inflammatory environment and enhancement of angiogenic properties (22).

Interestingly, not only MSCs but also their by-products are being tested for CKD. In 2016, Nassar et al. administered MSC-derived extracellular vesicles from CB-MSCs in patients

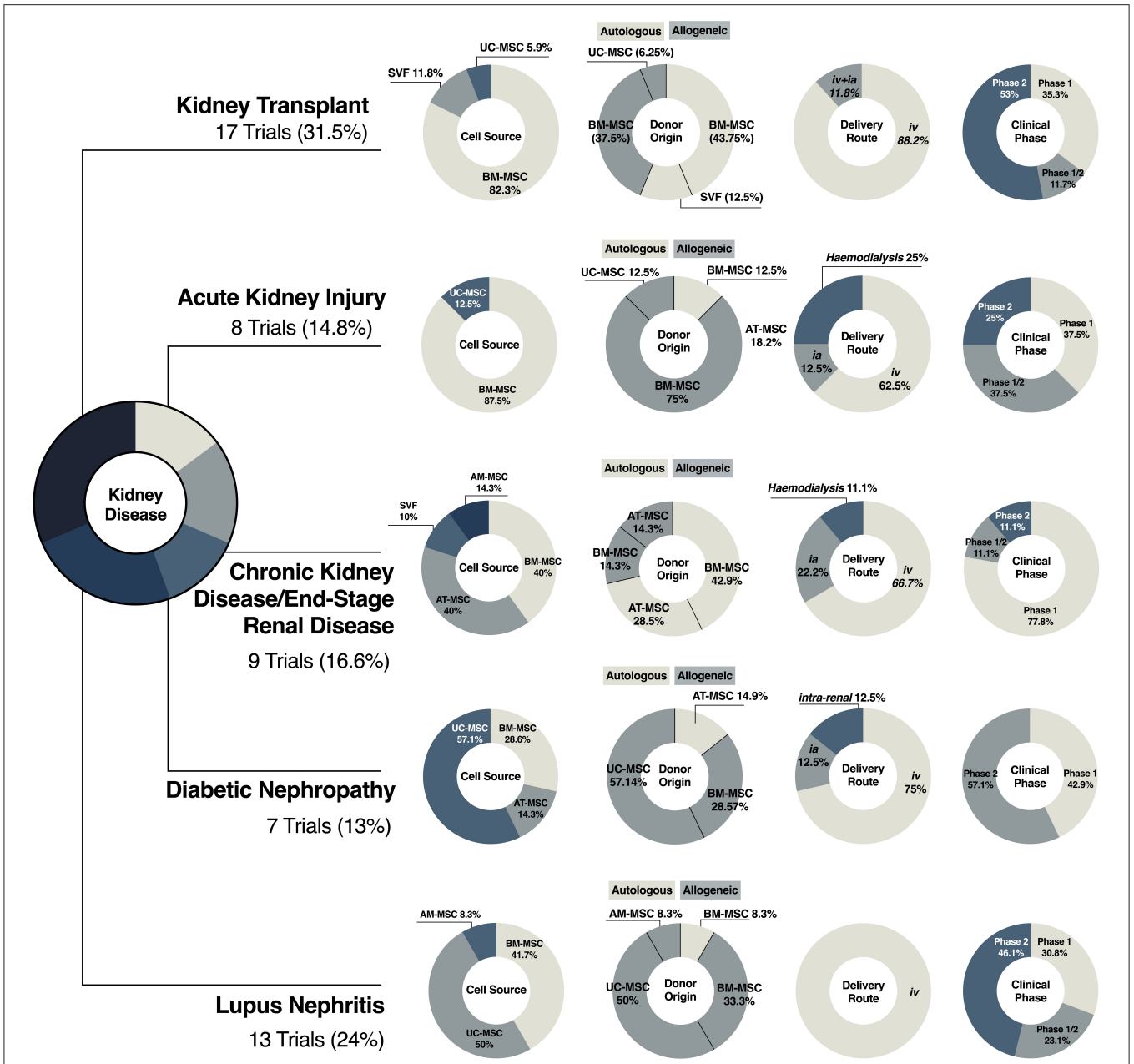

FIGURE 4 | Clinical translation of MSC therapies in kidney disease. Illustrative representation of the diversification of MSC-based products in clinical trials of kidney transplantation, acute kidney injury, chronic kidney disease, diabetic nephropathy and lupus nephritis, including cell source and donor origin, and clinical variables, such as delivery route and clinical phase in.

with CKD. Intravenous infusion resulted in significant improvement of renal function and increase in blood levels of immunoregulatory cytokines (203). Although preliminary, this study opens the window for novel strategies based on EVs derived from cultured MSCs with the potential of developing cell-free therapies (204).

In the context of CKD and ESRD, there is a tendency toward the use of autologous therapies, either from BM or AT. Although it has been recognized that MSC potency could be affected by disease status, several studies have supported the use of autologous strategies in CKD patients (205–207). Moreover, the use of allogeneic material is less desirable in patients likely to undergo renal replacement, as immune responses against donor antigens following MSC infusion have been documented (175). Overall, clinical protocols agree on administration route, frequency, and cell dosage (**Figures 3H–J**, **4**). It should be considered that underlying patient characteristics, such as disease stage, and the intrinsic complexity of chronic diseases could be reducing any therapeutic benefit.

Diabetic Nephropathy

Diabetic nephropathy (DN) is the most common cause of end-stage renal failure (208). In a multicentre, randomized, placebo-controlled, dose-escalation study conducted in Australia, the safety of two doses of allogeneic BM-MSCs product rexlemestrocel-L (Mesoblast Inc.) was tested in a cohort of patients with advanced DN. No adverse events related to the treatment were reported and patients showed trends of renal function improvement after 12 weeks (23). A similar phase I study conducted in 18 different centers across the USA in patients with type 2 diabetes, showed a decrease in glycated hemoglobin 12 weeks after infusion of the highest dose (209). Although the same MSC preparation and doses were used, improvement in glycaemic control was not observed in the Australian cohort. Both studies demonstrate that infusion of MSCs in diabetic patients is safe and well-tolerated; however, their results enhance the idea that therapeutic outcomes elicited by MSC transplantation may be largely influenced by the disease stage. Results from ongoing trials may be able to confirm the suggestive effects of MSCs in restoring renal function and potentially ameliorating biochemical alterations in DN patients.

Of interest, trials related to DN present a higher heterogeneity in choice of tissue source (five trials using UC-, two BM- and one AT-MSCs) but not in donor origin, with 85% of the trials using allogeneic sources, most likely because the impact of DM on MSCs is still under evaluation (210, 211) (**Figure 4**). Compared with other trials, protocols have included higher cell doses, with dose-escalation studies looking at fixed doses, and a range of administration frequencies, ranging from a single intravenous injection up to five doses over 32 weeks (**Figure 3I**).

Lupus Nephritis

Systemic lupus erythematosus is an auto-immune disease characterized by the loss of immune tolerance against self-antigens that affects tissues throughout the whole body (132). Lupus nephritis (LN) is the most common clinical manifestation (212). Considering the ability of MSCs to promote a tolerogenic environment and modulate the immune system, 13 clinical trials have explored the safety and efficacy of BM- or UC-MSCs in LN patients. In this particular scenario, therapies are mainly based on allogeneic cell products (**Figure 4**) due to impaired immune-modulatory properties and increased senescence in patient-derived BM-MSCs (213).

Results from successful trials identified serum levels of IFNγ as a predictive biomarker of MSC therapeutic efficacy. IFNγ have also been shown to stimulate the levels of IDO (214) and to have a critical role for UC-MSCs in regulating the innate immune system through up-regulation of tolerogenic dendritic cells (145).

However, conflicting results have been published reflecting the heterogeneity among SLE and LN patients, as well as the added challenge of intrinsic confounding factors such as different degrees of disease severity and treatment regimens. While a single intravenous administration of allogeneic BM- (24) and UC-MSCs (25) was proven to evoke clinical improvement and disease remission over time (215), other studies failed to reproduce the aforementioned results (26).

To date, there are 6 more registered trials where allogeneic BM- or UC-MSCs infusion have been or are being tested in safety and efficacy exploratory studies. Based on our search, similar therapeutic regimens—cell dose, frequency, and infusion route (**Figures 3I**, **4**)– have been or are being explored, with limited success when trying to increase infusion frequencies (28, 215) and adjust cell dose (29). Unfortunately lack of details about product preparation limit further inter-study comparisons.

BIOLOGICAL PROPERTIES OF MSCs DERIVED FROM DIFFERENT TISSUE SOURCES

The term "MSC" is nowadays used as an umbrella term that encompasses a variety of progenitor cells retrieved from a number of different tissues. This diversity has generated significant interest in further investigating the properties of MSCs derived from different in this review we have described the pre-clinical and clinical use of MSCs in kidney disease in which the cells have most often been harvested from bone marrow, adipose tissue and umbilical cord. It is not clear what is the rationale for use of MSCs from a particular source and if specific properties of the cells based on tissue of origin would suggest the superiority of one. In this section, we provide a comprehensive description of the biological and functional characteristics of MSCs reported in the literature depending on their tissue source to reflect further on this consideration (**Figure 2**).

Cell Morphology

MSCs are widely known for exhibiting a common spindle-shaped morphology, notably at the early stages of *in vitro* culture. Although this is the axiom from different starting tissues, cell size and morphology have been shown to vary between adult and younger sources, with perinatal-derived MSCs being relatively smaller and BM-MSCs cultures bearing heterogeneous populations (216–218). Morphological differences have also been reported between UC- and AM-MSCs even when the same genetic background was shared (216). Culturing conditions can also influence cell shape, as morphological changes have been attributed to different media compositions (219) and the use of specific supplements and growth factors (220). Besides altering lifespan, aging also influences cell morphology: older, senescent cells have a larger diameter (54, 221, 222). Interestingly, differences in cell size at early passage have been linked with differential expansion potential and senescence levels (223).

Growth Kinetics

MSC growth is characterized by an initial lag phase, where cells attach to the growing surface, followed by a log phase when cells undergo exponential growth by mitotic division. Finally, cells reach a plateau phase in which mitotic division continues but at a slower rate, as cell division is inhibited by cell-to-cell contact. This *in vitro* growth pattern continues at every passage until the hallmarks of replicative senescence start to appear, such as an increase in cell size, cell cycle arrest, interruption of mitotic divisions and accumulation of cellular debris and

stress fibers (224). For clinical and experimental purposes, MSCs must undergo *ex vivo* culture expansion to generate sufficient cell numbers. However, long-term culture expansion (or *in vitro* aging) has been shown to reduce the replicative lifespan and prompt the onset of senescence (54, 225, 226). This is an important fact, as it may limit the usefulness of these cells in cases where a high degree of *ex vivo* expansion is needed such as that required for achievement of clinical therapeutic doses. Thus, in a "space race" to discover which is the best MSC source for clinical applications, the ability to withstand longer periods in culture before reaching the onset of senescence is considered to be advantageous.

Many studies have now been performed that compare the expansion potential of MSCs obtained from different tissue sources, using culture parameters such as passage number, cumulative population doubling (CPD) and doubling times (DT) to describe cellular aging. When comparing the proliferation of MSCs harvested from different tissue sources, BM-MSCs have been shown to exhibit slower proliferation rates, with DT ranging from 40 to 60h depending on the culture conditions, and earlier appearance of senescence markers in relatively early passages (between passage 6 and 7) (217, 227–231). In contrast, AT-MSCs have shown faster proliferation rates (DT of 20 to 45h) as well as the ability to sustain a longer time in culture (up to passage 8) without any signs of senescence (217, 230, 232–234). These differences were still evident when comparing proliferation and differentiation capacity of AT- and BM-MSCs harvested from the same individual, although significant degrees of donor-to-donor variability was observed (232–234). Variables such as donor, age, sex, and disease status may have a significant effect on MSC characteristics (50, 51, 53, 235), which may discourage the use of adult sources as therapeutic agents while favoring MSCs obtained from birth-associated tissues (231). In general, these cells have exhibited higher proliferative kinetics with lower CPD over time (217, 230, 236–243), often related to lower expression of senescence-associated markers or later onset of senescence (229, 244), as well as upregulation of cell cycle-related genes and DNA damage response and repair (245, 246). These studies reflect the intrinsic heterogeneity between MSC populations in growth kinetics. Individual populations may also contain cells at different stages of differentiation and/or different proportions of highly proliferative cells. These variables have also been shown to vary from donor-to-donor (219).

Determining novel predictive biomarkers of therapeutic potency is of utmost importance before clinical usage, and viability and metabolic fitness have been recently proposed as potency qualities (184). Metabolic status is affected after long-term *in vitro* expansion, and it can reflect differential stemness behavior (242, 247–249), as well as cell immune functionality (250). Overall, considering the need to generate enough number of cells, the proliferative and metabolic characteristics of AT- and UC-MSCs may favor their use over BM-MSCs (251, 252).

Tri-lineage Differentiation Potential

The ability to undergo *in vitro* differentiation toward mesodermal lineages is, probably, the most differential property to biologically identify MSCs (253). Several culture-differentiating conditions have been reported to demonstrate the ability of MSCs to differentiate into adipocytes, osteoblasts and chondroblasts *in vitro*. Reports on tri-lineage differentiation potential have been inconsistent across different laboratories, and this may be due to the diversity of in-house protocols, culture conditions and media supplements, or the divergence in the cell preparations (219) and *in vitro* aging (54). Moreover, studies have reported a strong "tissue memory" effect, believed to be mainly driven by epigenetic factors (252, 254, 255). For instance, BM-MSCs present enhanced osteogenic and chondrogenic differentiation while AT-MSCs are usually more readily able to exhibit adipogenic differentiation (234, 241, 251).

Conflicting data however exists surrounding PT-MSCs as they have shown a heterogeneous potential to undergo mesodermal differentiation (241, 256, 257). Kern et al. reported that CB-MSCs could not differentiate toward adipocytes, similar findings were also reported for PL-MSCs (238). Other investigators, besides confirming the low adipogenic potential of CB-MSC (258), have reported higher osteogenic (247, 259) and chondrogenic potential (257, 260). Differences in identical genetic background perinatal MSC sources have also been described, with strikingly inconsistent results reported from AM-MSCs (216). Finally, similar observations have been reported for UC-MSC, with some studies suggesting higher adipogenic and osteogenic abilities (243), whereas others stated reduced differentiation compared with adult sources (240, 245).

While the field moves toward cell-free therapies (261) and mechanisms of action are mainly driven by paracrine and immunomodulatory effects (176), assessing the degree of commitment toward mesodermal linages to determine the most effective and suitable source for cell therapy may have less relevance. However, in other circumstances understanding how these differences affect the biology of MSCs could be an attractive avenue to study biological changes occurring throughout fetal development and adulthood (258), as well as to help define therapeutic strategies where use of MSCs is heavily influenced by such differentiation, such as bone and cartilage regeneration (262, 263).

Cell Surface Markers

MSCs are not a homogeneous population but rather an amalgamation of different subpopulations bearing different cell surface markers. Currently, a "true" marker for MSCs does not yet exist, which makes MSC identification challenging. In an attempt to unify MSC identification and characterization, in 2006 the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed a panel of minimal surface antigens to define human MSC (253). Within this criterion, they defined that at least 95% of the stromal population should express CD105, CD73, and CD90, and lack ($\leq$2%) the expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA class II. The negative markers are commonly used to confirm the absence of contaminant cells in MSC preparations such as hematopoietic progenitors, endothelial cells, leukocytes, and co-stimulatory molecules. The vast majority of studies have reported comparable immunophenotypic profiles that follow ISCT criteria regardless of source, although with moderate donor variability (219, 246,

264). In some cases, extended culture has been seen to reduce the expression of CD105 (54) and UC-MSCs have demonstrated lower (<95%) CD90 and CD105 expression (217).

However, the ISCT criteria do not uniquely identify stromal cells, as the proposed markers are also expressed in other connective tissue cells (265). Therefore, broader flow cytometry panels have been designed to best identify MSCs beyond the minimal criteria. Most of the protocols include the assessment of CD29, CD44, CD59, CD140b, CD166, TLR4, and PDL, commonly expressed (>95%) in human MSCs; and CD93, CD133, CD243, CD235, and SSEA1, with no or very low expression levels in human MSCs. Expression of other markers such as CD71, CD146, CD106, and CD274 has been shown to be heterogeneous, and in some cases correlates with donor age (53). Adhesion molecules such as CD44 (hyaluronic acid receptor) or CD29 (integrin β1 receptor) are highly expressed in human MSCs and have been recently proposed to be included in characterization panels (266). However, expression of markers such as CD146, another key adhesion molecule, can vary between sources, being highly expressed in UC-MSCs compared with BM-MSCs (230, 237, 240, 264) and subcutaneous AT-MSCs (267). Other markers found to be increased in UC-MSC preparations are CD10, CD49d (integrin α4), CD54 (ICAM1), (240) CD200, and PDL2, whereas CD119, IFNγR1 and CD183 (CXCR3) are under-expressed (264). An additional marker with functional relevance that has been shown to vary greatly between sources is the coagulation factor III or tissue factor (TF/CD142) (268), with increased levels being described in in AT- and PT-MSCs compared with those of BM-MSCs populations (170, 218, 269, 270).

Other researchers have investigated whether surface markers such as CD271, SUD2, MSCA1, CD34, and CD44 could serve to selectively enrich MSC populations. Differences between sources led to different selection efficiency and changes in biological properties. For instance, only CD34 was able to successfully isolate AT- and BM-MSCs, and interestingly the positive sorted populations showed greater proliferative capacity, increased osteogenic potential and HGF expression (271). Due to their perivascular origin, higher levels of CD34 and CD36 have been reported in AT-MSCs, albeit their expression decreases early after isolation (230, 234, 239). On the other hand, CD271 has been reported to be absent from MSC preparations in other studies (246, 272). Other differentially expressed markers are SSEA4 (higher in BM- and UC-MSC), MSCA1 (absent in UC-MSC, highly expressed in BM-MSC) and CD271 (high in BM-, low in AT-, absent in UC-MSC) (230, 271, 273). Nevertheless, it still remains unclear whether differences in MSC surface markers are correlated with therapeutic activity or potency (266).

Secretome Profile

It is now well-accepted that the therapeutic effects of MSCs are primarily mediated by their ability to interact and respond to environmental stimuli releasing soluble factors and EVs (274). The ability to sense changes is also translated *in vitro,* where cell culture conditions (219) or exposure to licensing strategies (275) can impact the secretome, highlighting plasticity and ability to adapt and respond to surroundings (274, 276). The so-called MSC secretome is composed of small molecules, chemokines, cytokines, growth factors, as well as EVs (277, 278). The literature has shown striking differences in the composition of MSC-secretome depending on the cell source. Moreover, variable results between studies add to the heterogeneity, further challenging the process of deciphering "true" biological properties that relate to therapeutic actions. It also makes it challenging to choose a specific MSC source to best align with the pathophysiology of the target disease.

Soluble Factors

MSCs have been reported to secrete large amounts of pro-angiogenic, pro-proliferative, anti-apoptotic, anti-inflammatory, anti-fibrotic and matrix-remodeling soluble factors. Several studies have shown that perinatal sources of MSC have a more diverse and protein-abundant secretome, with a more complete pro-angiogenic array (244, 246, 256, 279). Although some studies failed to detect differences in functional studies (256, 279), others have shown *in vitro* superior abilities of UC- and BM-MSCs in inducing angiogenic phenotypes (246). UC-MSCs have also exhibited greater abilities to induce vessel-like structures than maternal sources of MSCs, through enhanced secretion of HGF and VEGF (280). However, a potential confounding factor in these studies is the combination of maternal and fetal cells within PL-MSC preparations, which could be limiting their angiogenic properties (280).

In contrast with studies reporting that AT-MSCs had a weaker angiogenic secretome, lacking central molecules such as AKT1 and FGF2 (246), others have demonstrated *in vitro* and *in vivo* angiogenic potential of AT-MSC preparations in a model of hindlimb ischemia, due to the secretion of VEGFA, TGFβ, bFGF and HGF, well-known factors of endothelial cell survival, proliferation, and migration (251, 281, 282).

The secretome of UC-MSCs has been reported to be enriched with anti-inflammatory cytokines such as IL1RA and IFNα, pro-inflammatory cytokines such as IL6 and IL8; and mitogenic factors such as HGF, TGFβ2, PDGFAA and GCSF (240). BM-MSCs, on the other hand, while secreting lower levels of IL6, IL7, IL8, and IL12, have been reported to secrete higher concentrations of PDGFBB, MCP1, SDF1, TGFβ1, and VEGF (232, 240, 251, 283), exhibiting a stronger anti-inflammatory profile that increased upon exposure to hypoxic conditions, together with the expression of other angiogenic and anti-apoptotic factors such as ANG, HIFα, MMP9 and Bcl2 (284, 285). Increased levels of VCAM1 in the BM-MSC cytokine profile have been related to better angiogenic paracrine activity (275, 286).

AT-MSCs contain large amounts of IL7 and IL12 together with several metalloproteinases (MMP1, MMP3, and MMP13) and extracellular matrix components (240). Interestingly, expression of different MMPs between AT- and BM-MSCs has been previously reported, accounting for different mechanisms to promote angiogenesis (287).

Donor-to-donor variability and heterogeneity of MSC populations make it difficult to define a "secretome profile" specific for each tissue source of MSCs. Another layer of complexity relates to the use of cell culture supplements during *in vitro* expansion containing growth factors which may also

affect the secretome (246). Ultimately, dissecting the secretome of each specific MSC preparation may provide insights of their advantages in any given pathology (e.g., superior angiogenic secretome identified in BM- and UC-MSC preparations might make them an optimal source for ischemic disorders).

Extracellular Vesicles and miRs

In recent years, EVs have been proposed as a potential mechanism of therapeutic benefit of MSCs. EVs are lipid bilayer-delimited particles released by cells into the extracellular space carrying within them a range of cargos: subcellular components such as mitochondria, proteins, lipids, microRNAs (miRs), messenger RNAs (mRNAs) and transfer RNAs (tRNAs). Their roles have been described in multiple physiological and pathological process and are considered a mechanism of cell-to-cell signaling (288). MSCs secrete microvesicles (MVs) and exosomes, and both have been widely explored as cell-free alternatives to their cellular counterparts. Cell-free therapies, if able to recapitulate therapeutic efficacy of whole-cell preparations, offer several advantages due to a higher safety profile, lower immunogenicity, potential to bypass the lung trapping effect, and potential inability to induce neoplastic processes (289). It has also been described that EVs suppress pro-inflammatory processes, reduce oxidative stress and fibrosis in several *in vivo* models (290, 291).

Currently, there is limited data available on head-to-head comparisons of the paracrine benefits of different sources of MSC. We have only been able to identify a few studies reporting differential compositions and therapeutic effects of EVs derived from different sources. Exosomes derived from EM-MSCs enriched with miR-21 have been shown to confer superior cardioprotection after myocardial infarction over that of AT- or BM-MSC (292). Furthermore, a higher content of angiogenic-related cargos in EVs from AT-MSCs, compared to BM-MSCs, has been shown to promote wound healing (293). Similar findings have been attributed to the presence of miR-125a in AT-MSCs exosomes (294) and found to be enhanced by hypoxia priming (295). Albeit limited, recent data has described higher yields of particles secreted by AM-MSCs than BM-MSCs with similar size distribution, morphology, and immunophenotype (296).

Additional studies exploring the cargo within EV preparations from various sources have also reported beneficial effects. Exosomes secreted from BM-MSCs have been found to activate signaling pathways related to wound healing and angiogenesis (297–300) while their miRNA "repertoire" has been linked with anti-fibrotic, anti-apoptotic, pro-angiogenic and pro-proliferative properties (301) and the modulation of the native immune system (302). Exosomes originated from UC-MSCs have been found to contribute to wound healing (303) and reduce renal fibrosis after ischemic events by increasing capillarity density, reducing cell apoptosis, and restoring mitochondrial dynamics through miR-30b/c/d (116).

Despite the growing body of literature studying exosomes and their cargo in several settings, minimal evidence has been reported trying to underpin the molecular mechanism of action. Ferguson et al. investigated the biological processes modulated by exosomal miRs and found that targeted pathways were related to Wnt signaling, TGFβ and PDGF signaling, proliferation and apoptosis (301). Similarly, the expression profile of miRs in MSC-EVs derived from different sources lacks consistency. Although several studies have compared the expression between EVs and their parental MSC [reviewed by Qiu et al. (304)], limited studies have explored differences in miRs produced by different tissue-derived MSCs. To our knowledge, only one study has investigated the full RNAome derived from AT- and BM-MSC exosomes (305).

Akin to what we have described in the MSC field, EV isolation techniques lack standardization and generate variable products that can yield substantial differences. The use of serum or human platelet lysate supplements or serum-free conditions challenges the direct comparison of the relative contribution of EVs derived from MSC and other non-EVs factors. In a recent study, Whittaker et al. reported that soluble factors, that were non-EV molecules, were essential and sufficient to stimulate angiogenesis and wound healing *in vivo* (306). Their results concluded that most isolation techniques generate heterogeneous preparations containing other bioactive molecules that might mislead the attribution of therapeutic benefits.

Future studies defining the properties of miRs and exosomes will help in better understanding their biological functions and implications in cell-free therapies.

Immunomodulatory Properties

The immune system plays a central role in tissue recovery after injury. MSCs interact extensively with the immune system and promote an anti-inflammatory and pro-regenerative environment that favors injury resolution and, ultimately, tissue repair (19, 307, 308).

Many studies have demonstrated the ability of MSCs to modulate the activation, proliferation, and function of various immune cells such as T and B lymphocytes, natural killer cells (NK), dendritic cells, macrophages, and neutrophils. Such activities rely on the plasticity (309) of MSCs to produce cytokines in response to the different stages of the inflammatory process (310) and researchers are now investigating whether MSC immunomodulatory properties are influenced by their tissue of origin. Nevertheless, results from these studies are rather diverse and it is challenging to make adequate conclusions.

Some studies have compared the immunomodulatory properties of perinatal MSC, mainly UC-MSCs and CB-MSCs, with adult tissue sources (AT- and BM-MSCs). Overall, MSCs derived from perinatal tissues have the lowest expression of HLA antigens (HLA-DMA, HLA-DPB1 and HLA-DR) and immune-related genes (JAG1, TLR4, TLR3, NOTCH2, and NOTCH3) (243), together with decreased amounts of IL1α, IL6, IL8, (244) and increased IDO, IL1β, LIF, and TNFβ2 in their secretome (311). UC-MSCs also have the most prominent inhibitory effects on T cell proliferation, in both co-culture and trans-well mixed lymphocyte reaction (MLR) *in vitro* assays, followed by PL-MSCs, AT-MSCs and BM-MSCs (243). Other studies however have shown greater inhibition of allogeneic T cell proliferation by either BM-MSCs *via* increased expression

of PDL1, IL10, and TGFβ1 (230, 241), or AT-MSCs, which have been shown to secrete higher levels of IDO (243, 312).

Key adhesion molecules and other immunological markers such as CD10, CD146, CD49d, ICAM1 (CD200), and PDL2 are also increased in WJ-MSC preparations, together with decreased presence of IFNγR1, CXCR3 and other costimulatory molecules such as CD80, CD86, and CD40 (264, 313). In a recent *in vivo* study performed by Tago et al., AM-MSCs and not BM-MSCs were able to reduce local inflammation and $PD1^{+}CD8^{+}$T cell proliferation when delivered into a murine model of GvHD (314). In addition, PDL1-enriched EVs derived from UC-MSCs have been proven to be the mechanism whereby UC-MSC-EVs enhance immunosuppression (315).

In line with what has been briefly described, Mattar et al. have also highlighted the intrinsic heterogeneity of MSCs, where *in vitro* data also might not relate to the complex *in vivo* situation (316). The inflammatory context is defined by a variety of cell types and stimulating factors that are determined to influence and "license" MSCs which may adapt and change their interactions with the immune system as a result (310). Therefore, future studies should aim to decipher if similarities/disparities of *in vitro* results correlate to similar *in vivo* functions and whether biological properties can help to define cell performance, providing rationale for the use of one particular cell source for any given disease.

CONSIDERATIONS FOR THE GMP-PRODUCTION OF HUMAN MSCs FOR KIDNEY DISEASE

The regulatory agencies in the European Union (EU), United States of America (USA) and Asia, have adapted regulatory pathways to accelerate patient access to advanced therapies such as ATMPs. However, the legal frameworks for ATMPs, as well as the criteria to be met to define a product as such, differs across these regions (317–319). In Europe, MSC-based ATMPs are governed by the EU Regulation 1294/2007/EC and Directive 2009/120/EC, and its manufacturing must be compliant with European current good manufacturing practice (cGMP) guidelines (EudraLex Volume 4, Part IV). These ATMP-specific regulations have been put in place to ultimately ensure the safety and wellbeing of patients, as single alterations in the bioprocess hold the potential to alter the final product with potential risk to the patient. For this reason, in the absence of proof of product comparability, regulatory authorities are prompted to require further re-validation, which in the worst-case scenario have resulted in pre-clinical data being invalidated and clinical trial approval requiring re-authorization. Thus, optimal manufacturing variables must be considered and identified during early stages of development, as changes in the bioprocess workflow later in the translational pathway may have a significant long-term impact on the success of the therapy with time and cost consequences but also a significant time-to-market delay (320, 321). In addition, having full control of the process is crucial for ensuring consistent production and quality standards in terms of safety, identity, and potency, and this control can only be guaranteed by having in place systems of quality assurance (QA) and quality control (QC) across all stages of the bioprocess. While guidelines and some common criteria now exist for ATMP developers to follow, consistent clinical-grade production of MSCs is not yet achieved due to a lack of standardization and harmonization of manufacturing processes. Critical parameters in MSC manufacturing include the source of the starting material, and culture processing conditions, such as seeding density, passage number, media supplements and culture-expansion devices, among others (322, 323). These parameters have been shown to be highly variable among manufacturing centers and laboratories worldwide (47–49), increasing the number of variables, as illustrated in **Figure 5**, that should be considered when carving therapeutic approaches in specific clinical settings such as KD.

Tissue Origin

For many years BM has been the predominant cell type in clinical trials for kidney disease (**Figure 3D**) and others (47, 48, 324). One of the main advantages of using BM-MSCs is the ability to use them in autologous settings without triggering anti-donor immunoreactions. However, donor-related parameters such as age, disease severity and presence of comorbidities should be considered as they have been shown to affect MSC characteristics (53, 235, 325, 326). Recent attention has been given to donor gender, as there is now increasing evidence of gender influencing MSC properties such as growth kinetics, paracrine secretion and *in vivo* therapeutic potential (52, 53, 327, 328). Another disadvantage of using BM-MSCs is the need for large amounts of raw material to allow for extensive *ex vivo* cell expansion to obtain clinical doses, as these cells constitute a rare population (only 0.001–0.1%) within the whole bone marrow fraction (329). Finally, BM collection requires an invasive bone harvest procedure, which is accompanied by pain, risk of infection and other limitations such as patient's comorbidities that can render this procedure unsuitable.

Alternative sources such as AT have been considered for many years. Subcutaneous AT has been shown to be an abundant source of AT-MSCs, with a yield of MSC precursors 500 times higher than from an equivalent amount of BM (330). AT-MSCs can be used both in autologous and allogeneic settings, which is advantageous. In addition, AT can be easily accessible as it is discarded as medical waste in many operations, which would be useful in allogeneic settings. In autologous settings, AT is harvested by less invasive procedures than BM, such as liposuction. However, like in BM sources, the therapeutic efficacy of autologous MSC therapies may be limited by the intrinsic impact that the disease, age, and gender may have on MSCs characteristics (50, 51).

Thus, in the past few years, more consideration has been given to the potential use of allogeneic MSC therapies due to the hypoimmunogeneic phenotype of these cells (331). Perinatal and birth-associated tissues have become an attractive source of allogeneic MSCs for many reasons, the main being that this material is considered medical waste and discarded every day in hospitals worldwide. Also, these MSCs are obtained from

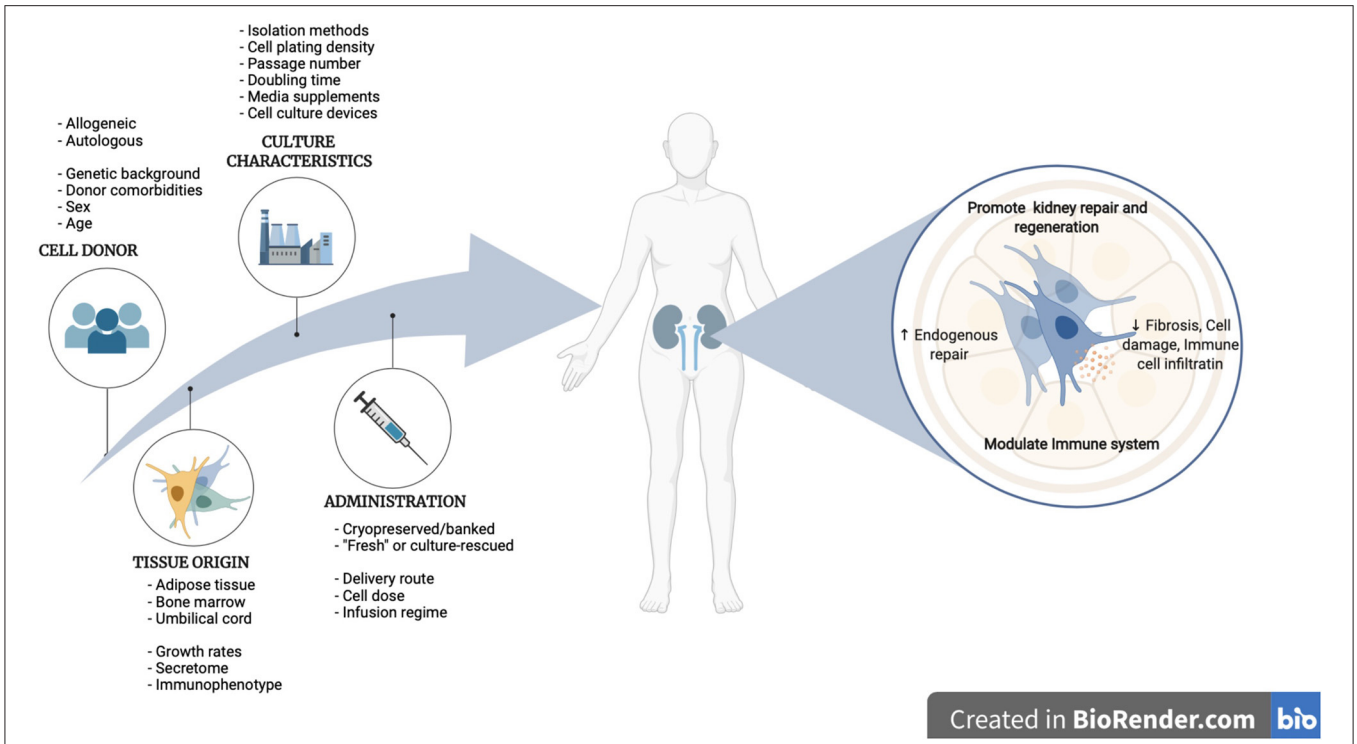

FIGURE 5 | Roadmap to clinical translation of MSC therapies for kidney disease, from MSC manufacturing variables to therapeutical benefits in renal pathologies.

the youngest donors possible (neonates), removing donor age-related confounding effects. Perinatal tissue sources such as UC have reported isolation rates ranging from 0.2–1.8% (216), and these cells have also been shown to have significantly higher proliferation rates, compared to MSCs isolated from adult tissues (217, 237, 241).

Overall, results from our search showed that the predominant source of MSCs used in clinical trials in renal pathologies is BM (58.1%), followed by UC (23.6%) and AT (11%) (**Figure 3D**). Also, autologous MSC therapies are predominant (62.5%) over allogeneic. Similar trends have been also observed in other studies (47, 48, 324). Interestingly, in renal pathologies, BM-MSCs have been mostly used in allogeneic settings (59.4%), while AT-MSCs have been used mostly in an autologous manner (80%) (**Figures 3E**i–iii).

Culture Processing Characteristics

Based on ongoing clinical trial data in kidney disease, human MSCs are transplanted at typical doses of 1–2 million cells/kg and often not exceeding 10 million cells/kg (**Table 2**). Doing a basic dose extrapolation, for an 80 kg person the estimated human MSC doses per patient would range between 80 and 800 million cells per patient. Thus, the generation of clinical doses of MSC requires large-scale *ex vivo* cell expansion and having an optimal scale-up strategy for MSC manufacturing is critical for ensuring product quality while minimizing costs and time of production, as well as avoiding potential risks. The following are some key variables in the cell culture bioprocess to consider when designing MSC therapeutics.

Cell Plating Density

Cell plating density is a key parameter to ensure adequate expansion rates while maintaining stemness properties (332). The literature suggests that plating densities, both at isolation and subculturing, can influence functional and molecular characteristics of the MSCs (49, 219, 333), and yet it is something not well-standardized across laboratories. There are contradictory reports regarding the optimal subculturing seeding densities. Generally, higher plating densities (i.e., >5000 cells/cm^2) have resulted in reduced proliferation rates, most likely due to contact inhibition by confluency and the need for continuous premature passaging (332), which is known to critically affect the proliferation rate of MSCs (242, 333–337). Also, the log phase has a longer duration in cells plated at low densities, and therefore more population doublings occur due to a longer exponential growth phase (338). Thus, finding the optimum seeding density for a maximal expansion of therapeutic MSCs while being cost-effective is crucial (339). Some studies have recommended using very low seeding densities when subculturing, as such required for clonal selection (i.e., <500 cells/cm^2), as it has been shown to result in the highest cell proliferation rates (339–342). Other studies have used slightly higher densities between 2, 000 and 4, 000 cells/cm^2 (49, 206, 343). The disadvantage of using very low seeding densities (i.e. <100 cells/cm^2) for a clinical-scale production of MSCs is the large surface area required to culture therapeutic doses of MSCs, which is not feasible when using 175 cm^2 flasks, due to the need for large incubator occupancy, a sizable amount of lab reagents and increased handling times. Plating densities of 1, 000 cells/cm^2

have been considered reasonable, as this density still allows for a high number of harvested cells (243, 342, 344). However, often more cost/labor compromises are undertaken with most current clinical trials using plating densities of over 3, 000 cells/cm^2 (48). In the kidney disease clinical trial arena, a mix of low and high plating densities have been reported, ranging from 100 cells/cm^2 to 500, 000cells/cm^2 (**Table 2**).

Cell plating densities at the isolation phase have also shown similar outcomes in clinical trials. Sotiropoulou et al., have shown that initial plating density of bone marrow mononuclear cells (BM-MNCs) had a great impact on the size of the MSC-enriched population derived, with the maximum number of adherent cells at P0 obtained when using lower plating densities (<25, 000 cells/cm^2) compared to high plating densities (>50, 000 cells/cm^2), with 1, 000 cells/cm^2 being the optimal condition (341). But similar challenges are encountered here, where large surface areas may be needed for the initial plating. For instance, given that up to 1×10^8 BM-MNC are commonly obtained, $\sim$600 $\times$ 175 cm^2 would be needed to seed 1×10^8 BM-MNC at 1, 000 cells/cm^2, which is not practical or cost-efficient. Indeed, the most common seeding densities used in clinical trials are 1.5–1.6 $\times$ 10^5 cells/cm^2, followed by 1×10^6 cells/cm^2 (48). In our search on clinical trials for kidney disease, seeding densities at isolation have been reported to be 1–2 $\times$ 10^5 cells/cm^2 (**Table 2**). One approach to reducing the plating surface area at isolation would be cell enrichment by prospective immunoselection using antibodies directed against specific cell surface markers to obtain a more homogeneous, pure, and well-defined functional subset of MSC subpopulation. For instance, some markers that have been used to purify distinct subsets of MSCs include CD146 (345), CD271 (346, 347), Stro-1 (348) and CD362 (349), which have shown properties such as having greater paracrine immunomodulatory and anti-inflammatory properties (188, 345, 349), increased osteogenic commitment (346, 347), and higher production of cardiovascular-relevant cytokine production (348).

Passage Number

MSCs are an adherent cell population and have normal growth inhibition when confluent. This has led to the use of successive passages for obtaining a large amount of MSCs. Passage number, which refers to the number of times cells have been sub-cultured, is often recorded as an indicator of cellular age. Cell expansion requires enzyme dissociation and cell subculture, and while the evaluation of the optimal cell confluence may vary among operators, a 70–80% confluence is recommended to be reached before detachment (323). In general, passage numbers from 1 to 5 are commonly used in clinical trials (48, 324) and we have also found similar trends in our search (**Table 2**). The use of low passage MSCs for therapy is currently preferred to higher passages due to the impact that extended passaging has in decreasing the cell proliferation rates and increasing senescence times (242, 333–337). Long-term culture has also been shown to affect other properties of MSC such as immunosuppressive activity (242, 335), trilineage differentiation (333, 334, 337), *in vivo* therapeutic potential (333, 337, 350), and have also been shown to increase genomic instability although not to induce *in vivo* tumorigenicity (336, 337). Also, the advantage of transplanting MSC at earlier passages over late passages was demonstrated clinically in patients with GVHD, where 1-year survival rates were higher in those patients that received MSCs at passages 1–2 (75%) compared to those that received later passage MSCs (passage 3-4) (350). Effects of passage number in combination with cryopreservation cycles have also been described to impact the safety profile of MSC products, with cells cultured for extended times triggering stronger prothrombotic events compared with cells cultured for shorter times and "fresh" cells (44, 168). For these reasons, regulatory agencies have recognized the importance of cellular age tracking during the manufacturing process, as well as the need for karyotypic analysis as a product release criterion. While passage number has been traditionally used for cellular age tracking, this is largely dependent on the specific seeding and harvest density conditions, and therefore it is challenging to make comparisons between studies. Population doubling level (PDL), which refer to the total number of times the cells have doubled during *in vitro* culture, is, therefore, a more robust and accurate parameter to define cellular aging. An upper limit of PDL, before culture ceases to replicate, must be defined for each product. The literature suggests a maximum number of cell population doubling to be between 15 and 30 (333), although this may be influenced by the cell type (i.e., UC-MSC showed higher proliferation rates and later senescence than AT- and BM-MSCs) (230, 242, 243, 351) and the culture processing characteristics (219).

Media Supplements

The most common basal media employed in current MSC expansion protocols are DMEM or αMEM (48), although αMEM has shown to be more suitable for both isolation and expansion of MSCs (341, 352). MSCs however require media supplements such as serum and/or growth factors to be added to the basal medium for optimal MSC growth. Most expansion protocols, especially at laboratory-scale and in early phase clinical trials, have used fetal calf serum (FCS) with 10% being the standard concentration used for MSC expansion (48). Basic fibroblast growth factor (b-FGF), at a final concentration of 1-2 ng/ml, is also added to the basal media to enhance the proliferation rate of cultured cells while maintaining the multilineage differentiation potential (353, 354). Nevertheless, the use of FCS for large-scale production of clinical doses of MSCs is not a viable option (355). Limitations in the availability of the raw material are a major cost driver and represent a current bottleneck. Also, due to the batch-to-batch variability, FCS lots must be carefully tested to ensure optimal MSC expansion rates and tri-lineage differentiation potential. In addition to this, there are current regulatory challenges associated with the use of FCS to produce clinical-grade MSCs due to the risk of inter-species cross-contamination, and regulators urge the development of xenogeneic-free compositions. Considerations to using human-derived blood components such as human platelet lysate (hPL), often at a final concentration of 5–10%, have been given (47, 356). While hPL has been shown to have comparable growth factors and cytokines to FCS to support MSC growth (355), it poses some important challenges (357). HPL can be derived from autologous

collections, but it does not represent a good commercial model. Large scale, allogeneic, off-the-shelf pools are easier to standardize, have less lot-to-lot variation, is more economical to produce and therefore represents a better commercial model. Nevertheless, while hPL collections can be obtained from up to 100 different individuals, the size of pools is a current issue and a topic of debate. Recently, regulators have expressed their concern about the increased risk of transmission of infectious agents in large pooled hPL products. The *European Pharmacopeia* have recommended the limitation of pooled donations unless sufficient methods for inactivation or removal of viruses are applied during the production, although it does not give specific recommendations to the pool size (Chapter 5.2.12). However, representatives of the German Federal Regulatory Authority specified the restriction to a maximum of 16 donors (358). This imposes many challenges for ATMP developers and commercial entities, who must fast adapt their products to the constantly evolving regulatory framework. Overall, a consensus is needed to ensure the quality and safety of hPL supplements regarding the source of platelet concentrate, donor- and lot- variability, manufacturing processes and minimum release criteria (357).

Due to the concerns mentioned above, the development of new xenogeneic-free, chemically defined formulations is urgently needed. Chemically defined media (CDM) are generally composed of basal media to which supplements of known composition (i.e., growth factors, hormones, attachment factors, binding proteins, and vitamins) are added (320). An ideal MSC media should contain chemically defined constituents preferably of recombinant human origin that support the isolation and culture expansion of human MSCs obtained from different tissue sources while maintaining MSC phenotypic characteristics, morphology, and mechanism of functional benefit. Ideally, it should also support the attachment of MSCs without coating. Extensive testing is however required to ensure these media fulfill MSC requirements, but when successful, this type of media will have the potential to enhance batch-to-batch consistency in the cell manufacturing process and will therefore represent a more cost-effective and risk-reduced approach. To date, 10% FCS continues to be the most common media supplement employed in clinical trials for kidney disease, although some consideration has been given to the use of xenogeneic-free media such as hPL (5 or 10%), human serum albumin (HSA) and a CDM (**Table 2**). Currently, some commercial and non-commercial CDM formulations have been investigated (359–363), however, there is still limited availability of some of these media for large-scale manufacturing at GMP quality level.

Cell Culture Devices

Currently, a complete closed system that allows MSC acquisition, expansion, and delivery at the bedside, is not yet available. GMP conditions have been mainly achieved using laminar airflow cabinets to perform the main steps in the bioprocess such as culture inception, medium changes, subculturing and packaging. Traditionally, scale-out of MSC manufacturing has been achieved using 2D monolayer cultures using multilayered flasks (Corning® CellSTACK and Nunc™ Cell Factory™) of 1 to 40 levels, and surfaces ranging from 636 cm to 25, 440 cm. However, this is not an optimal system for large-scale production of therapeutic doses, as it is labor-intense, requires significant manual handling, and is not cost-effective (320, 364). These are also static systems, which lack real-time process monitoring of culture conditions, and are more susceptible to batch-to-batch variation due to a non-homogeneous environment within layers (320). Alternatively, GMP-compliant, closed, automated, high-volume cell expansion systems offer great advantages, as they enable the real-time monitoring of process variables such as pH, pO_2, pCO_2, metabolite accumulation or presence of contaminants, and hence it guarantees a homogeneous distribution of culture environment and ensures a culture process under well-controlled and reproducible conditions and production of quality MSCs for clinical use. A variety of bioreactors are available including stirred tank bioreactors with microcarriers (365, 366), rocking motion (367), disposable fixed bed (368), and hollow fiber-based continuous perfusion bioreactors (369, 370). The surface area from these bioreactors ranges from 21, 000 cm^2/unit to up to 3, 750, 000 cm^2/unit, and they all offer distinct advantages and limitations that must be considered (320, 364, 371). In our search, an overwhelming majority of clinical trials have used 2D culture conditions, with only 1 study considering a 3D bioreactor, the Quantum Cell Expansion System (Terumo BCT) (NEPHSTROM clinical trial, NCT02585622) (**Table 2**).

CONCLUSIONS AND FUTURE PERSPECTIVES

While the field of cell-based therapies evolves, the selection of particular MSC types in specific clinical conditions remains to be elucidated. In the past decades, BM has been the preferred source of MSCs used in clinical trials of kidney disease, but recently allogeneic sources have emerged as strong candidates in the clinical research arena. Ideally an allogeneic, "off-the-shelf" MSC product would be preferred, especially for acute kidney disease settings where delivery time is crucial. We hypothesize that, the use of MSCs may be rationalized by the intrinsic origin-specific properties which may make one cell type more advantageous for a specific disease condition. Overall, the high proliferative capacity, the stronger immunosuppressive effects and hypo-immunogenetic properties of UC-MSCs paired with their allogeneic nature makes them ideal to be used in an "off-the-shelf," large-scale, universal production model. Although it is likely that the choice of MSC type may be driven by intellectual and/or industrial property on isolation methods, protocols and/or reagents in addition to issues of functional and biological superiority, considerations should be also given to the safety of this therapies, in particular accounting for differences in immune and hemocompatibility characteristics. Considering this constellation of variables, robust clinical guidelines and well-characterized therapeutic products are urgently needed to deliver safer, effective, and potent MSC therapies to improve clinical outcomes. This will require a greater understanding of the biology of MSCs from different tissue sources along with an alignment with disease pathophysiology coupled with

consideration and standardization of the cell manufacturing variables reviewed in this article.

AUTHOR CONTRIBUTIONS

SC-C, CS-N, and TO'B wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by grants from SFI CÚRAM Research Center (Grant No. 13/RC/2073), Science Foundation Ireland (SFI) (Grant No. 18/IF/6252) and from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie Grant Agreement No. 813839.

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Conflict of Interest: TO'B is a founder, director and equity holder in Orbsen Therapeutics Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Medicine

REVIEW
published: 13 October 2021
doi: 10.3389/fmed.2021.728543

iPSC-Derived Organoids as Therapeutic Models in Regenerative Medicine and Oncology

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Progress made during the last decade in stem cell biology allows currently an unprecedented potential to translate these advances into the clinical applications and to shape the future of regenerative medicine. Organoid technology is amongst these major developments, derived from primary tissues or more recently, from induced pluripotent stem cells (iPSC). The use of iPSC technology offers the possibility of cancer modeling especially in hereditary cancers with germline oncogenic mutations. Similarly, it has the advantage to be amenable to genome editing with introduction of specific oncogenic alterations using CRISPR-mediated gene editing. In the field of regenerative medicine, iPSC-derived organoids hold promise for the generation of future advanced therapeutic medicinal products (ATMP) for organ repair. Finally, it appears that they can be of highly useful experimental tools to determine cell targets of SARS-Cov-2 infections allowing to test anti-Covid drugs. Thus, with the possibilities of genomic editing and the development of new protocols for differentiation toward functional tissues, it is expected that iPSC-derived organoid technology will represent also a therapeutic tool in all areas of medicine.

Keywords: induced pluripotent stem (IPS) cell, organoid, cancer, drug discovery, regenerative medicine

OPEN ACCESS

Edited by:
Debbie Guest,
Royal Veterinary College (RVC), United Kingdom

Reviewed by:
Andreas Kurtz,
Fraunhofer Society (FHG), Germany
Sridhar Goud,
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *21 June 2021*
Accepted: *17 September 2021*
Published: *13 October 2021*

Citation:
Turhan AG, Hwang JW, Chaker D, Tasteyre A, Latsis T, Griscelli F, Desterke C and Bennaceur-Griscelli A (2021) iPSC-Derived Organoids as Therapeutic Models in Regenerative Medicine and Oncology. Front. Med. 8:728543. doi: 10.3389/fmed.2021.728543

INTRODUCTION

Organoids are tridimensional assembly of cells, mimicking organ-like features generated *in vitro* under specific cues (1). Their ability of self-organization *in vitro* under defined conditions allows their development for several days or weeks depending on the conditions of culture. There are now extensive data showing that these structures can recapitulate some of the features observed in adult organs, opening therefore major perspectives for their use in disease modeling, precision oncology and perhaps in the future as tools of regenerative medicine. This technology has also the potential to replace animal experiments as theoretically any tissue can be generated *in vitro*.

Currently, organoid-like structures have been successfully generated from several human tissues (1). These include essentially heart, digestive system, liver, brain, lung, and kidney organoids. These structures have been used to generate either healthy or diseased tissues, allowing to compare the behavior of different cell populations contributing to the organoid under *in vitro* conditions. They

have provided important clues for the identification of new signaling pathways and novel targets especially in precision oncology. However, they require obviously a biopsy which might be difficult to obtain. The advantage of these organoids generated from cancer tissue biopsy is obviously the possibility of obtaining more precise information with regard to the tumor microenvironment as this is discussed below. On the other hand, primary cancer-derived organoids are not amenable to genome editing or extensive long-term cultures. One approach to circumvent these obstacles and which is currently in development is the use of iPSC technology to manufacture different organoids which is the subject of this review.

IPSC-DERIVED ORGANOIDS

The pioneering work of S. Yamanaka which led to the revolutionary iPSC technology allows the reprogramming of an adult somatic cell toward an embryonic state similar to embryonic stem cells (ESC). Since their initial description in 2006 in mice (2) and in 2007 in human cells (3), iPSC are increasingly studied in stem cell research and more recently in the therapeutic arena by the possibility of generating differentiated cells for therapeutic purposes (4). More recently, the attention was focused on the possibility of generating from pluripotent stem cells (either iPSC or ESC) organ-like structures called "organoids" initially described from the adult tissue samples (1).

The organoid field has emerged from pioneering work of the group of Hans Clevers which has shown initially the possibility of generating gut organoids from Lgr5+ stem cells (5). These findings have now been reproduced and extended to other tissues and organoids have been obtained from several adult tissues. More recently, IPSC-derived organoids came into the forefront of stem cell research, due to the fact that as compared to adult tissue-derived organoids, they offer the possibility to combine the self-organization potential of iPSCs and the possibility of directing these cells toward potentially to any organ-specific differentiation (6).

Based on the events of human embryonic intestinal development, Spence at al first showed the possibility of generating intestinal organoids using a series of successive growth factor additions allowing endoderm induction, patterning and morphogenesis from human embryonic stem cells (H1, H9) and from four lines of iPSC. Interestingly, the 3D intestinal structures showed functional features of intestinal epithelium such as absorption and exocrine functions (7). Similarly, using self-organizing embryonic stem cells, Nakano et al. (8) have shown the possibility of generation of 3D optic cups.

in vitro models of brain development have also been possible with the advent of iPSC-derived organoids technology. In 3D culture systems, it was possible to generate mini-brains with highly specialized zones and structures such as cortex and radial glial cells and to model human microcephaly (9). The initial methodology has now progressed to the stage where it is possible to generate highly specialized cells such as oligodendrocytes and astrocytes (10) as well as long-term culture procedures of cerebral structures leading to highly specialized advanced brain organoids to study later stages of neural development (11). Finally the possibility of generating separately different parts of human brain has been described (12).

In the field of kidney development and kidney diseases, iPSC-derived kidney organoids allowed the possibility of generating highly specialized structures such as distal and proximal tubules as well as glomeruli with podocytes with a transcriptomic features similar to that of human fetal kidneys (13). iPSC-derived kidney organoid technology is of interest not only for gaining pathophysiologic insights but also to determine the effects of drug development in the transplant setting, for instance to evaluate the toxicity of tacrolimus (14).

The complex structure of the liver can also be reproduced using iPSC-derived organoid technology. One of these studies has shown the possibility of obtaining transplantable 3D hepatic buds with functional activities by the combined culture of iPSC-derived endoderm directed toward hepatic differentiation along with mesenchymal stem cells and the endothelial HUVEC cell line (15) Hepatic organoids can also be obtained from normal or patient-derived iPSC to model human hepatic diseases (16, 17). Although adult liver tissue can be targeted to generate hepatic organoids, iPSC-derived liver organoids can have a potential advantage of their expansion ability, which can be of interest for toxicology purposes allowing to screen large numbers of compounds in the industrial setting (17). ESC and iPSC-derived cardiac structures can be obtained with highly reproducible methods, giving rise to contractile structures including the possibility of morphological compartmentations such as cardiac chambers (18). This technology represents also an important tool for drug screening but also for disease modeling using patient-derived iPSC (19) (**Figure 1**).

The multicellular nature of the lung can also be recapitulated using IPSC-derived organoids (20) allowing generation of 3D structures containing lung progenitors, alveolar type 2 (AT2) cells as well as airway cells and alveolar macrophages (21). In this field, the organoid technology has been used to model hereditary lung diseases such as cystic fibrosis with demonstration of gene correction potential (22).

It therefore appears that the unique pluripotent nature of iPSC is a major asset for the generation of organoids-in-a dish toward any types of structures with advantages but also disadvantages as compared to adult-tissue-derived organoids, as summarized in **Table 1**.

Finally, this highly sophisticated technology with several steps of culture may hold promise not only for the study of infectious diseases (such as SARS-Co-V2) but also for therapeutic purposes as an ATMP product (see below).

ORGANOIDS AS ATMPS: HYPE OR HOPE?

Given the differentiation potential of iPSC toward almost any organoids, the next question is their potential use as ATMPs. Large scale cGMP grade production of organoids could also lead to the possibility of manufacturing "transplantable" organoids and tissues in the future. From this regard, cardiac and liver tissues could be candidates be generated for transplantation

TABLE 1 | Comparative features of adult tissue vs. iPSC-derived organoids.

Origin of organoid	Availability	Protocols	Differentiation potential	Potential use as ATMP*	Cancer organoid models	Utility in cancer immunology research	Potential for use in infectious diseases	Genome editing	Organ-on chip studies	Potential biobanks	Challenges ahead in 2021
Adult-tissue-derived	Requires tissue and biopsy	Relatively easy	Depends on the type of organs and stem cells	Limited	High potential	Yes, TME** with immune components are present	Limited (Requires tissue sampling)	Limited	Yes	Yes	Improved differentiation protocols
											Ability to expand
iPSC-derived	Readily available from iPSC	Complex and multistep procedures	Theoretically unlimited towards any tissue	High potential	High potential but TME absent.	TME components are not reproduced in a single step	High Potential	High potential	Yes	Yes	Simplified differentiation protocols
					Interest in hereditary cancer studies						Adequate vascularization and innervation

*Advances therapeutic medicinal product.
**Tumor micro-environment.

purposes. However, in this field, many efforts are underway and many obstacles remain to be solved. If the use of IPSC-derived corneal cell transplantation has already began in a trial in Japan (23), iPSC-derived organ transplantation in humans is currently a long way from clinical applications but there are studies showing the functional cells can be manufactured. Indeed, in experimental conditions, it has been shown that iPSC-derived kidney nephron structures improve acute renal failure in mice (24). Similarly, erythropoietin-(EPO)-producing iPSC)-derived nephrons could improve anemia associated with terminal kidney failure (25).

In lung diseases such as idiopathic pulmonary fibrosis where the only cure is the lung transplantation, the possibility of generating and transplanting iPSC-derived autologous alveolar epithelial cells could have a significant impact on the prognosis (26).

In the field of diabetes, the transplantation of iPSC-derived islet organoids could be of therapeutic interest in the future as this has been validated in experimental setting (27).

What are the challenges lying ahead before the clinical applications? The vascular organization of future organoids is a major challenge but from patient-specific IPSC, it could be possible to generate after imprinting a brain or heart organoid containing microvasculature derived from HUVEC cells (28).

Similarly, it is necessary to provide in the organoids of the future an adequate innervation system. The possibility of generating iPSC-derived intestinal tissues with an enteric nervous system has been described, generated by combing human intestinal organoids and pluripotent-stem cell derived neural crest cells (29). A combined IPSC-derived hierarchized organoids called "assembloids" have also been described in the hepatobiliary system, generated by inducing the fusion of anterior and posterior gut spheroids, leading a hepato-biliary pancreatic organoids (30). Transplantation of these structures into immunodeficient mice failed however, to give rise to a multi-organ differentiation (30).

IPSC-DERIVED ORGANOIDS IN CANCER

In the field of cancer, the possibility reproducing cancer of a given organ could be of substantial interest, especially to develop drug screening and for precision medicine. From this regard, cancer organoids could allow to capture genetic heterogeneity as well as the progression-related modifications in a given cancer. Using adult-tissue derived organoids, several studies have shown that organoids generated from the initial tumor as well as from their metastatic counterparts match closely with the original tumor in breast cancer (31). Established from the initial tumor biopsies before any therapy, these organoids arising *in vitro* within 1–3 months could serve as an *in vitro* drug screening tools (32). One major drawback of this technology is the fact that it requires obviously the availability of a tissue sample which is not always possible. The growth of the structure is also limited as the tumor biopsy does not always recapitulate the hierarchical subtypes of a tumor. On the other hand, the major advantage of this approach as compared to IPSC-derived modeling is the

possibility to capture the cellular components of the tumor microenvironment, including immune competent and immune-suppressive cells which could allow the potential responses to immune therapies such as check-point inhibitor therapies (see **Table 1**).

Although more challenging as compared to adult tissue-derived cancer derived organoids, iPSC-generated cancer organoids can be of interest in the study of patients with hereditary cancers. iPSC technology allows also the *de novo* generation of cancer organoids using genome editing (**Figure 2**). In the unique situation of the context of hereditary cancers, especially in the carriers of the oncogenic mutation with no established cancer, it may be possible to generate iPSC and to use this oncogene-bearing cell line as an organoid specific of the target tissue such as breast cancer or kidney cancer. This approach was first reported by the group of I. Lemischka using IPSC derived from patents with Li-Fraumeni disease (33).

In our group, we have generated c-MET-mutated iPSC from a patient with hereditary papillary renal cell carcinoma (34). We have shown that kidney organoids generated *in vitro* recapitulate the transcriptomic features of the primary PRCC of a large cohort of patients. The target genes that we have identified have been also confirmed in the kidney biopsies of patients with PRCC (34). The presence of a given oncogenic mutation in an iPSC line, allows the evaluation of the phenotype generated upon differentiation toward a given pathway and model therefore cancers occurring in several different tissue lineages. We have thus asked whether c-MET mutated iPSC could allow modeling glioblastoma, a tumor in which an overexpression of c-MET has been described in 10% of cases. We have showed that neural structures derived from these iPSC exhibit transcriptomic features close to that observed in human GBM (35).

The iPSC technology offers also the possibility of generating organoids after induction of specific genomic modifications using molecular manipulation of iPSC (**Figure 2**). An oncogenic mutation which can be induced in the pluripotent state, can then be propagated with generation of a "transformed organoid" as this has been shown for modeling glioblastoma (36). More recently, it has been shown that overexpression of KRAS G21D oncogene in pancreas acinar cells allow development of pancreas cancer *in vitro* (37) Similarly, recent work showed the possibility of generating in iPSC-derived RAS-mutated alveolar type 2 (AT2) cells, the induction of a genomic pathways similar to lung adenocarcinoma which is a driver gene in 30% cases (38). This study allowed to determine the early genomic changes occurring in the AT2 cells some of which were similar to transcriptomic features of in primary lung adenocarcinoma such as overexpression of Sox9 (38). Thus, iPSC-derived cancer organoid technology is expected to expand during the next decade, with several models developed for other cancers (39, 40) with increasing implication of microfluidic technology to study drug sensitivity (41). These technologies will also benefit from the molecular analyses and bioinformatics techniques with discovery of novel targets leading to therapeutic intervention (34, 42).

USE OF IPSC-DERIVED ORGANOIDS IN INFECTIOUS DISEASES

Organoids appear today as major experimental tools for determining target cells and pathophysiology of viral infections. This concept has been successfully applied to Zika virus (43–45) and more recently to SARS-Cov-2 infections. In fact, in a very short period of time, the availability of these bioengineering tools led to their exponential use for several research teams worldwide to identify target cells for COVID-19 entry, and to evaluate the potential therapeutics, and vaccine approaches.

Using a large number of organoids derived from iPSC, it has been shown that the organoids such as pancreas are highly permissive to the virus (46).

Tiwari et al. have generated iPSC-derived brain and lung organoids to study the virus entry into different cell populations and their differential responses to COVID-19 infection (47) In particular they have shown that as compared to lung organoid derived cells, neural progenitors and astrocytes expressing low levels of ACE2 were not permissive to COVID-19, a finding that has been documented in pluripotent stem cells (47). The iPSC-organoid technology represents also a major tool to study drugs that best allow inhibition of virus entry into cells of different origin (48). Human IPSC-derived organoid technology allows also, by the combined use of CRUSPR CAS9 techniques, to determine the genetic susceptibility of human populations to Covid_19 (49). Indeed a unique sNP found in the 3′UTR of the Furin gene has been shown to influence the infection of lung and neuronal cells by Covid 19. These technologies could therefore help to define populations which could be an increased risk for Covid 19 infection (49). One other interesting potential use of organoid technology in the field of infectious diseases is the use of gastric organoids to study the infection by Helicobacter Pylori (50, 51).

PERSPECTIVES AND CHALLENGES AHEAD

Organoid technology, developed initially from the primary normal or diseased tissues/organs has achieved a novel major perspective by the use of iPSC technology, which offers the possibility of genomic editing and theoretically an unlimited proliferation and differentiation potential. As summarized, the technology holds tremendous potential but several hurdles remain, explaining its current limitations. For larger medical applications, better differentiation protocols are needed. The fact that cells organize themselves in 3D conditions suggest that some cell to cell interactions must occur to lead to pre-organoid structures called 'aggregates" and to the phenomenon of symmetry breaking which occurs during normal embryonic development.

One other limitation of iPSC-derived organoids is the fact that they do not represent the typical environments which are usually found in tissues, especially in cancer in which a particular immune-suppressive microenvironment is present. Similarly, the application of this organoid technology in the future kidney

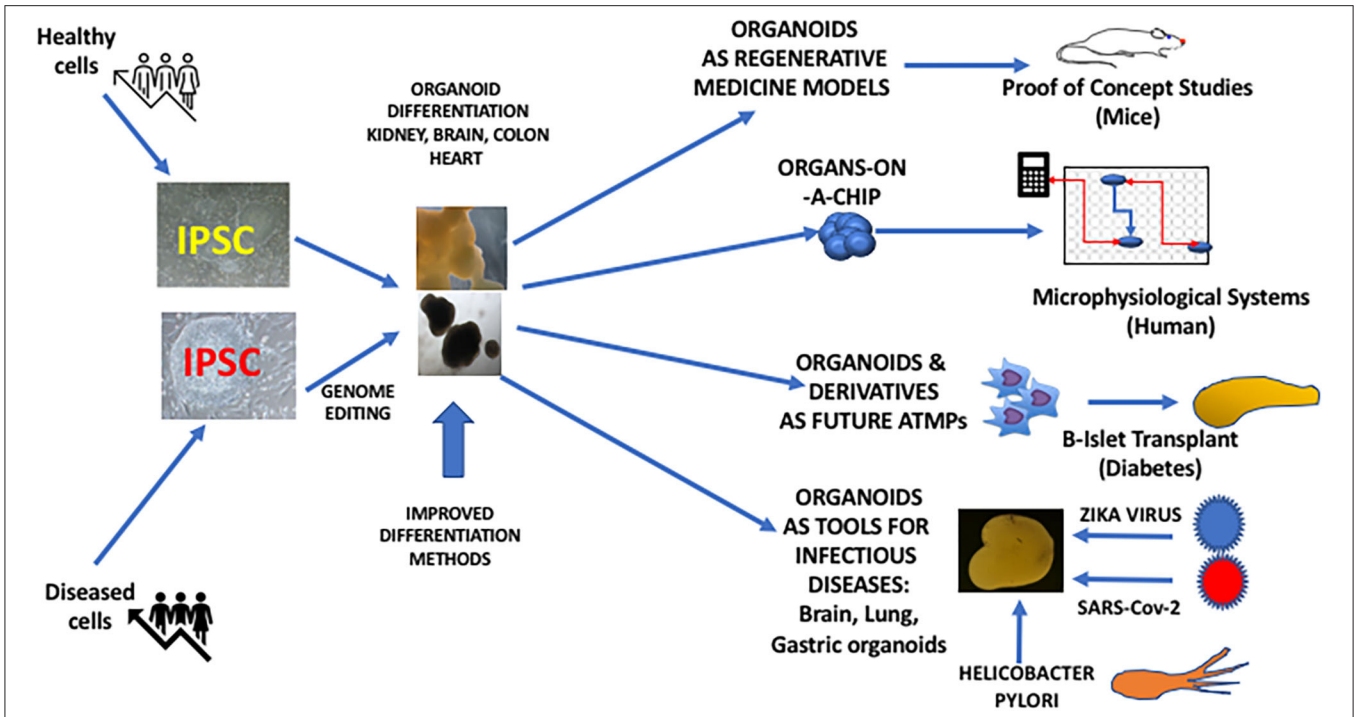

FIGURE 1 | iPSC-derived organoids and their potential use in Regenerative Medicine.

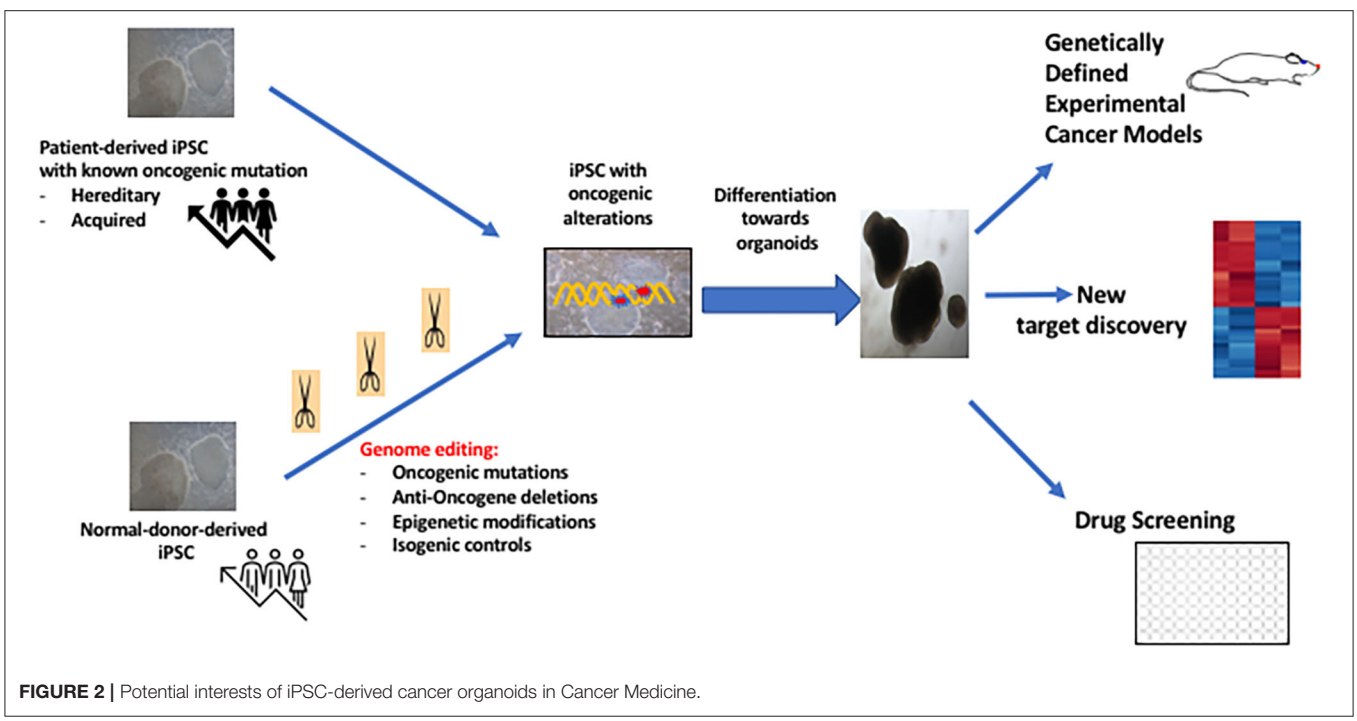

FIGURE 2 | Potential interests of iPSC-derived cancer organoids in Cancer Medicine.

transplants, will require the possibility of generating a functional vasculature but also a urine drainage system, which is not yet been achieved.

Thus, the generation a functional vasculature within the *in vitro* generated organoids is a significant challenge. Similary, organoids might miss some changes related to aging of the organ especially when generated from iPSC. A recently described human Organoid Atlas could be of major help to generate a "catalog" for human organoids including the standardization of their methodology. Single cell transcriptome analyses as well as spatial profiling will be of major areas of research during the next decade.

A H2020 project is currently in progress project within the human cell atlas project (https://hca-organoid.eu).

Finally some ethical issues might need to be discussed in the future with regard to the generation and the use of reprogrammed cells but also with regard to the creation of complex and increasingly sophisticated iPSC-derived human organoids (52). Indeed, such ethical issues will need to be discussed for example, with the possibility of generating complex brain organoids, complex integrated systems or early developmental structures such as amniotic sacs (53). These complex integrated systems have already been developed using microphysiological systems allowing organoids to be used for emulation of human biology in "human-organs-on-chips" systems and will pave the way for the drastic reduction of animal use for drug discovery experiments (54, 55).

Overall, both adult tissue and iPSC-derived organoids offer an unprecedented complementary information in almost all areas of medicine with accelerated discovery of novel targets and potentially as a therapeutic ATMP modality in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was performed by the internal funds of INGESTEM.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 26 October 2021
doi: 10.3389/fmed.2021.739987

RESTORE Survey on the Public Perception of Advanced Therapies and ATMPs in Europe—Why the European Union Should Invest More!

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Advanced therapy medicinal products (ATMPs) are potential game changers in modern medical care with an anticipated major impact for patients and society. They are a new drug class often referred to as "living drugs," and are based on complex components such as vectors, cells and even tissues. The production of such ATMPs involves innovative biotechnological methods. In this survey, we have assessed the perception of European citizens regarding ATMPs and health care in Europe, in relation to other important topics, such as safety and security, data protection, climate friendly energy supply, migration, and others. A crucial question was to determine to what extent European citizens wish to support public funding of innovations in healthcare and reimbursement strategies for ATMPs. To answer this, we conducted an online survey in 13 European countries (representative of 85.3% of the entire EU population including the UK in 2020), surveying a total of 7,062 European citizens. The survey was representative with respect to adult age groups and gender in each country. Healthcare had the highest ranking among important societal topics. We found that 83% of the surveyed EU citizens were in support of more public funding of technologies in the field of ATMPs. Interestingly, 74% of respondents are in support of cross-border healthcare for patients with rare diseases to receive ATMP treatments and 61% support the reimbursement of very expensive ATMPs within the European health care system despite the current lack of long-term efficacy data. In conclusion, healthcare is a top ranking issue for European Citizens, who additionally support funding of new technologies to enable the wider application of ATMPs in Europe.

Keywords: advanced therapies, advanced therapy medicinal products (ATMPs), healthcare, European Union, survey in Europe, public perception

OPEN ACCESS

Edited by:
Ondrej Viklicky, Institute for Clinical and Experimental Medicine (IKEM), Czechia

Reviewed by:
Jadranka Buturovic Ponikvar, University Medical Centre Ljubljana, Slovenia
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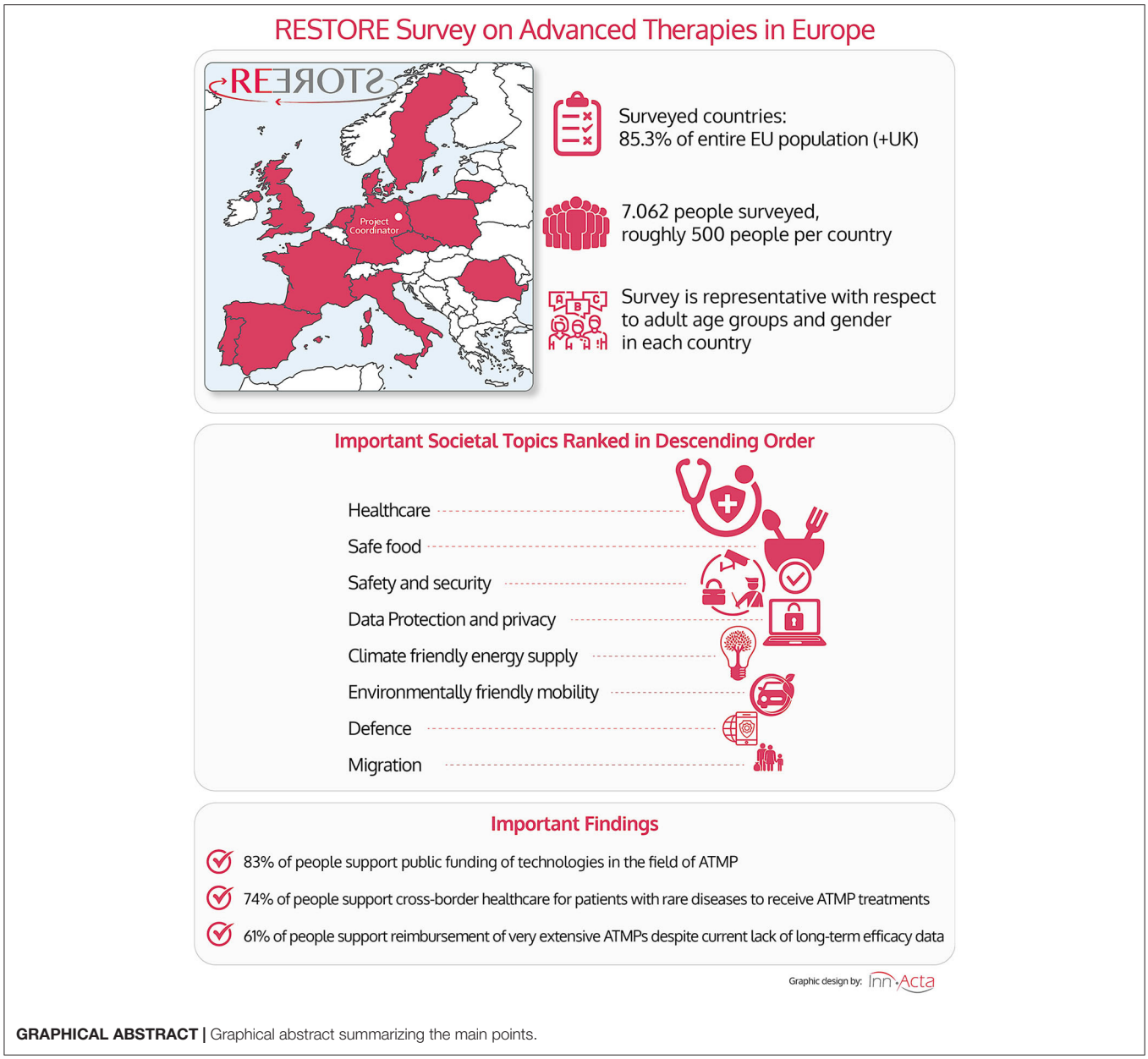
Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *12 July 2021*
Accepted: *23 September 2021*
Published: *26 October 2021*

Citation:
Goldsobel G, von Herrath C, Schlickeiser S, Brindle N, Stähler F, Reinke P, Aberman Z, Ofir R, Dessole G, Benvenuti S, Neves NM, Reis RL, Moll G and Volk H-D (2021) RESTORE Survey on the Public Perception of Advanced Therapies and ATMPs in Europe—Why the European Union Should Invest More! Front. Med. 8:739987. doi: 10.3389/fmed.2021.739987



GRAPHICAL ABSTRACT | Graphical abstract summarizing the main points.

INTRODUCTION

Advanced therapies or advanced therapy medicinal products (ATMPs) are innovative medicinal products that include gene therapies, cellular therapies, and tissue engineered products (1). These new therapeutic approaches aim to repair or replace lost function, thereby generally aiming for long-term effects or even cures instead of symptomatic treatment. Several major reviews and surveys considering the development of ATMPs in Regenerative Medicine (RegMed), Tissue Engineering (TE), and Stem Cell (SC) industry have been done in the past, mainly focusing on the dominant United States (US) market (2–4). The core of our current survey was to assess the perception of the European public toward their opinion and support for ATMPs in European health care.

ATMPs can be tailored with great precision to specific treatment indications. These can be both common pathologies, but also very specific indications with a so far unmet medical need, provided the clinical benefit, price, and regulatory requirements warrant the effort (5–13). Indeed, ATMPs may range from very cost-efficient and broadly marked "off-the-shelf" therapeutics for common diseases and pathologies, to highly specific, but in this case more costly, therapeutics that target otherwise difficult or impossible to treat indications, as is

often the case with genetic defects and rare diseases. However, a number of practical and administrative hurdles need to be overcome before ATMPs can be integrated into the existing health care ecosystems and be made broadly available to the public. This may entail novel funding concepts and enabling an organized development of manufacturing technologies and production sites, as well as new reimbursement strategies.

To generate and employ ATMPs, special manufacturing technologies and facilities are needed to fulfill the high regulatory quality standards required for clinical development and for their marketing. This entails advanced biotechnological methods, good manufacturing practice (GMP) facilities and integrated clinical database approaches, all of which can be quite costly in their use and thus require appropriate reimbursement strategies and constant innovation to lower the costs for these innovative treatments (6, 14–17). Highly promising recent developments in the field demonstrate the therapeutic potential of ATMPs, for example products, such as chimeric antigen receptor (CAR)-based T cell therapies for hematological/oncological malignancies and gene therapies for rare diseases (16, 18–20). While this has led to an increased use of ATMPs in medical practice, technical, financial, and regulatory issues still prevent their more widespread implementation into standard medical care.

It is of principle importance to understand the opinion of patients, their health care providers, and the public as a whole, regarding their potential support of this important new field in Europe and globally, so that ATMPs will not only become a niche-product, but a strong pillar of modern health care (6–9). To assess the public opinion of European citizens, this survey was conducted in the context of the European Union/Commission (EU/EC)-funded RESTORE large-scale research initiative to further promote the development and use of ATMPs in Europe (https://www.restore-horizon.eu/). The unifying goal of RESTORE is the implementation of newly developed Advanced Therapies in clinical routine to improve patients' outcome with high impact on Europe's society and economy.

We here aim to determine and understand the public opinion on ATMPs in the EU, a major economic region, especially with regards to questions related to public funding of research in the field and government reimbursement of ATMPs. We believe that this information will provide useful insights into public perception and therefore provide guidance for future policy decisions.

METHODS

The questionnaire was first developed in English and then translated to all languages of the surveyed countries. Every question also included a short explanation of the topic in layman terms (**Table 1**). The questionnaire was transferred into an online format for distribution and sampling of respondents was conducted by 4C Consumer Insight GmbH in the form of an online survey from a large cohort of potential participants of whom around 80% who agreed to participate also completed the survey.

TABLE 1 | Structure and explanation of surveyed questions.

(x) General assessment of important social topics

(A) Knowledge about ATMPs prior to survey

1. Did you hear about ATMPs prior to your participation in the survey?

This question had an introductory purpose to assess the level of knowledge prior to participation in the survey.

2. Did you notice a recent trend of ever increasing number of approved ATMPs and ATMPs in clinical trials?

Research with ATMPs has been around for many decades, but a real awakening has only occurred in recent years with the introduction of very effective ATMPs to the market. Professionals in the field have observed this trend, but we were interested to see whether the general public also observed this re-emergence.

3. Have you heard about private clinics that offer non-approved ATMPs to patients?

The phenomenon of private clinics offering unapproved therapies is not new or unique to the field of ATMP, but professionals in the field have noticed a trend of increasing number of clinics offering non-approved ATMPs and we were interested to see whether this trend caught the attention of the general public.

4. What do you consider to be an appropriate measure to prevent private clinics from administering non-approved ATMPs to patients?

Here, we aimed at getting the public's opinion whether this phenomenon should be fought, if at all, with hard measures such as tight enforcement of the law or softer measures such as a warning from the media.

(B) Opinions on public funding in healthcare

1. Please rate on a scale of 1–5 the following topics: healthcare, climate friendly energy supply, data protection/privacy, migration, safe food, IT-infrastructure, safety and security, environmentally-friendly mobility, sustainable use of natural resources and defense.

Here, we aimed at identifying the public's perception on the importance of healthcare, compared to other important societal topics, where R&D is often also publicly funded.

2. Do you think EU- and state-funding should be invested in the development of future medical innovations?

The question did not pertain specifically to ATMPs but to medical innovations in general. The question served as an introduction to the following question as it helps identify and illustrate how the opinion of the survey participant changes from general medical innovations to ATMPs.

3. Should EU and Member States fund enabling technologies for cell and gene therapies?

We asked about public's support of funding of R&D in technology and material related to ATMPs. This is important, as it helps to identify what medical innovations EU citizens are interested in and how future government budget should be allocated. It also helps to identify whether education and awareness raising activities are required.

(C) Opinions on reimbursement of ATMPs

1. Should the state pay for expensive therapies although evidence for long-term benefit has not been shown yet?

Here, it was important to us to ask a balanced question by giving an accurate description of current scientific facts. To achieve that, we explained and emphasized the lack of long-term efficacy data for currently available ATMPs.

2. Do you agree that, in the case of rare diseases, cross-border health care (e.g., traveling abroad) is the best way to provide the most beneficial treatment for patients?

ATMPs are complex products that can often only be administered by specialists in dedicated treatment centers. For rare diseases, the number of patients is often low and it is not possible to open dedicated treatment centers in every region or country. Statutory coverage of cross-border healthcare would mean the taxpayer funds treatments given in another European country. We aimed to find out if European citizen support this reimbursement concept.

3. Should non-medical costs be covered in cross-border healthcare?

This question pertained to one of the big hurdles in reimbursement of cross-border healthcare: If medical treatment is administered abroad, non-medical costs (such as cost of travel and accommodation) are not usually covered by health insurers. We were interested in knowing the public's opinion on the possibility of reimbursing non-medical costs, by law, in case of cross-border treatment.

TABLE 2 | European populations, and per capita, relative and total healthcare spending (the surveyed European countries are marked in red/orange; currency Euro, EUR).

Country	Annual healthcare spending per capita (Status 2018 in EUR)	Population (1st of Jan 2020)	Relative population (% of EU Total)	Healthcare spending (National in EUR)	Relative HC spending (% of EU Total)
Austria	4,501	8,901,064	1.73%	40,063,689,064	2.53%
Belgium	4,150	11,549,888	2.25%	47,932,035,200	3.02%
Bulgaria	587	6,951,482	1.35%	4,080,519,934	0.26%
Cyprus	1,645	888,005	0.17%	1,460,768,225	0.09%
Czech Republic	1,493	10,693,939	2.08%	15,966,050,927	1.01%
Germany	4,627	83,166,711	16.17%	384,812,371,797	24.27%
Denmark	5,256	5,822,763	1.13%	30,604,442,328	1.93%
Estonia	1,312	1,328,976	0.26%	1,743,616,512	0.11%
Greece	1,320	10,709,739	2.08%	14,136,855,480	0.89%
Spain	2,310	47,329,981	9.20%	109,332,256,110	6.89%
Finland	3,829	5,525,292	1.07%	21,156,343,068	1.33%
France	3,969	67,098,824	13.05%	266,315,232,456	16.79%
Croatia	862	4,058,165	0.79%	3,498,138,230	0.22%
Hungary	917	9,769,526	1.90%	8,958,655,342	0.56%
Ireland	4,613	4,963,839	0.97%	22,898,189,307	1.44%
Italy	2,634	60,244,639	11.71%	158,684,379,126	10.01%
Lithuania	1,061	2,794,090	0.54%	2,964,529,490	0.19%
Luxembourg	5,221	626,108	0.12%	3,268,909,868	0.21%
Latvia	936	1,907,675	0.37%	1,785,583,800	0.11%
Malta	2,290	514,564	0.10%	1,178,351,560	0.07%
Netherlands	4,480	17,407,585	3.38%	77,985,980,800	4.92%
Poland	830	37,958,138	7.38%	31,505,254,540	1.99%
Portugal	1,877	10,295,909	2.00%	19,325,421,193	1.22%
Romania	584	19,317,984	3.76%	11,281,702,656	0.71%
Sweden	5,041	10,327,589	2.01%	52,061,376,149	3.28%
Slovenia	1,831	2,095,861	0.41%	3,837,521,491	0.24%
Slovakia	1,100	5,457,873	1.06%	6,003,660,300	0.38%
United Kingdom (UK)	3,646	66,650,000	12.96%	243,005,900,000	15.32%
Total	**2,604**	**514,356,209**	**100%**	**1,585,847,734,953**	**100%**
(*n* = 28)	(Mean value)	(514 million)	(Relative to Total)	(1,6 million million)	(Relative to Total)
Participants without UK	**2,847**	**372,458,152**	**72%**	**1,160,838,997,572**	**73%**
(*N* = 12; Red Only)	(Mean value)		(Relative to Total)		(Relative to Total)
Not participating with UK	**2,423**	**141,898,057**	**28%**	**425,008,737,381**	**27%**
(*N* = 16; Black+Orange)	(Mean value)		(Relative to Total)		(Relative to Total)
Participants with UK	**2,908**	**439,108,152**	**85%**	**1,403,844,897,572**	**89%**
(*N* = 13; Red+Orange)	(Mean value)		(Relative to Total)		(Relative to Total)
Not Participating without UK	**2,341**	**75,248,057**	**15%**	**182,002,837,381**	**11%**
(*N* = 15; Black only)	(Mean value)		(Relative to Total)		(Relative to Total)

Green is high contribution or percentage and red is low contribution or percentage.

The list of countries included those from Northern, Southern, Western, and Eastern Europe, including countries that founded the EU and newcomers. It included both wealthy and less wealthy countries (**Table 2**), with an average "Per Capita" annual healthcare spending representative/similar to the European average with or without UK inclusion, covering 514 million inhabitants (before UK exit) with a total budget of 1.6 million-million Euros. The survey took 4 months to complete with ∼1 week per country. While eight countries (DE, FR, UK, IT, ES, PL, PT, and NL) were surveyed in January 2020, before COVID-19 was declared a pandemic, the remaining five countries (CZ, DK, LT, RO, and SE) were surveyed amidst the COVID-19 lockdown period in Europe, until end of April 2020. This may have had a potential impact on the public perception of some of the topics proposed. However, when analyzing the data we did not observe a clustering of answers related with the first or second group of countries.

Additional information was collected regarding the educational qualification of the surveyed people, their income level, their status of employment and the number of inhabitants in their locality. With the exception of one question, participants could answer on a scale from 1 to 5, where 1 means "yes,

definitely" or "very important" and 5 means "definitely not" or "very unimportant." For the analysis we used the average weighted response for each question. When examining the data with Excel there did not appear to be any missing data or outliers. Regarding representability, the cohort was representative in respect to the sex and the age groups in each of the surveyed countries. However, it is not representative in respect to other criteria. This may present a deviation from the true numbers. However, to see how strongly these deviations change the data we normalized the data in respect to the education level of the surveyed citizens. This normalization did not change the results. Finally, we took a deliberate choice to include the same number of surveyed people from each country, although their sizes varied greatly. To address this potential limitation, we normalized the data with respect to the size of the countries. Again, the normalization did not change the results.

RESULTS

Introduction of the Study Design

As summarized in Graphical Abstract, the survey was conducted online with a total of 7,062 citizens, interviewed from 13 European countries (roughly 500 people per country), including the Czech Republic (CZ), Denmark (DK), France (FR), Germany (DE), Italy (IT), Lithuania (LT), The Netherlands (NL), Poland (PL), Portugal (PT), Romania (RO), Spain (ES), Sweden (SE), and the United Kingdom (UK). On the 1st of January 2020, these countries accounted for 72% of the EU population (without the UK) or alternatively 85% when including the UK (**Table 2**). The survey has been designed to be representative with respect to adult age groups and gender in each country.

To structure the survey, we formulated 10 major questions covering three main aspects on ATMPs (**Tables 1A–C**), containing: (A) Knowledge about ATMPs prior to initiation of the survey, (B) Opinion on public funding of research, and (C) Opinion on reimbursement issues.

Due to the complexity of the issue and for better comprehensibility, we chose to first present the data on the general assessment of important social topics in the population (The first question of topic B: question B-1) in **Figure 1** before addressing the more detailed data from part A–C in the **Figures 2–4**. A summary of the interrelationship of different factors presented in **Figure 5**, which was reproduced according to a prior design by Aiyegbusi et al. first presented in their review "Patient and Public Perspectives on Cell and Gene Therapies" (21).

Assessment of Important Social Topics

We first wanted to obtain an impression how European Citizens view the importance of "Healthcare" compared to other important societal topics, which require European funding, e.g., "Migration," "Safe Food," and "Climate Friendly Energy Supply," as summarized in Graphical Abstract and **Figure 1**. Although all these issues were rated to be important (average scores ranging between 1.3 and 2.6 on a scale of 1–5), on average, the by far most important issue for the European citizen was healthcare (Graphical Abstract central section and **Figure 1A**), with the topic "Healthcare" scoring on average two times higher than the lowest scoring issue "Migration" in our survey (**Figure 1B**). The topic "Healthcare" was followed by the general importance of "Safe Food," "Safety and Security," and "Data Protection/Privacy," while environmental issues (e.g., Sustainable use of natural resources and climate friendly energy) took surprisingly only a middle ranking (**Figure 1B**). Interestingly, "IT-infrastructure" and "Defense" ranked rather low, in the range of "Migration." It is worth noting that "Healthcare" already scored highest with citizens surveyed before the COVID-19 pandemic outbreak.

We furthermore studied how was this affected by population age (**Figure 1C**), population size (**Supplementary Figure S1A**), educational qualification (**Supplementary Figure S1B**), income status (**Supplementary Figure S1C**) and employment status (**Supplementary Figure S1D**). Importantly, the same order of ranking was observed, regardless of the aforementioned factors. Regarding population age (**Figure 1C**), older age groups gave more importance to all topics than younger ones. There was only little influence of population size with great homogeneity of results in differently sized European countries (**Supplementary Figure S1A**). People with the highest educational qualification level (PhDs) attached less importance to topics than people with lower educational qualifications (**Supplementary Figure S1B**). In addition, people with lower income gave more importance to all topics than people with higher income (**Supplementary Figure S1C**). Regarding employment status, retired people gave more importance to all topics than students (**Supplementary Figure S1D**). This is in line with the observation concerning population age. One exception to the order of ranking can be seen with CEOs of large companies (all topics seem to have the same importance). However, this group consists of only 91 people and may not be large enough to draw major conclusions about the views of this group in general. Overall, healthcare clearly stands out as the most important topic in our survey of European citizens.

Knowledge About ATMPs Prior to Survey

A key component of the survey was to assess the general knowledge of EU citizens on ATMPs prior to the survey (**Figure 2**), which was structured into four questions (**Table 1A**). The introductory question "Have you heard about ATMPs before?" was answered positively by 50–70% of people (lowest in Germany and highest in The Netherlands, European average 58% Yes to question A-1) (**Figure 2A**). Although ATMPs have been around for many decades, a real awakening only occurred in recent years. We thus asked next, "If participants noticed a recent trend for increasing numbers of approved ATMPs and their clinical trials?," which was answered positively by between 30 and 70% of participants (lowest in Germany and highest in Spain, European average 55% Yes to question A-2) (**Figure 2B**). The phenomenon of private clinics offering unapproved therapies is not new or unique to ATMPs, but professionals in the field have noticed a trend of increasing numbers of clinics offering non-approved ATMPs and we were interested to see whether this trend caught the attention of the general public. We thus asked: "Have you heard about private clinics offering non-approved ATMPs to patients?" which was answered positively

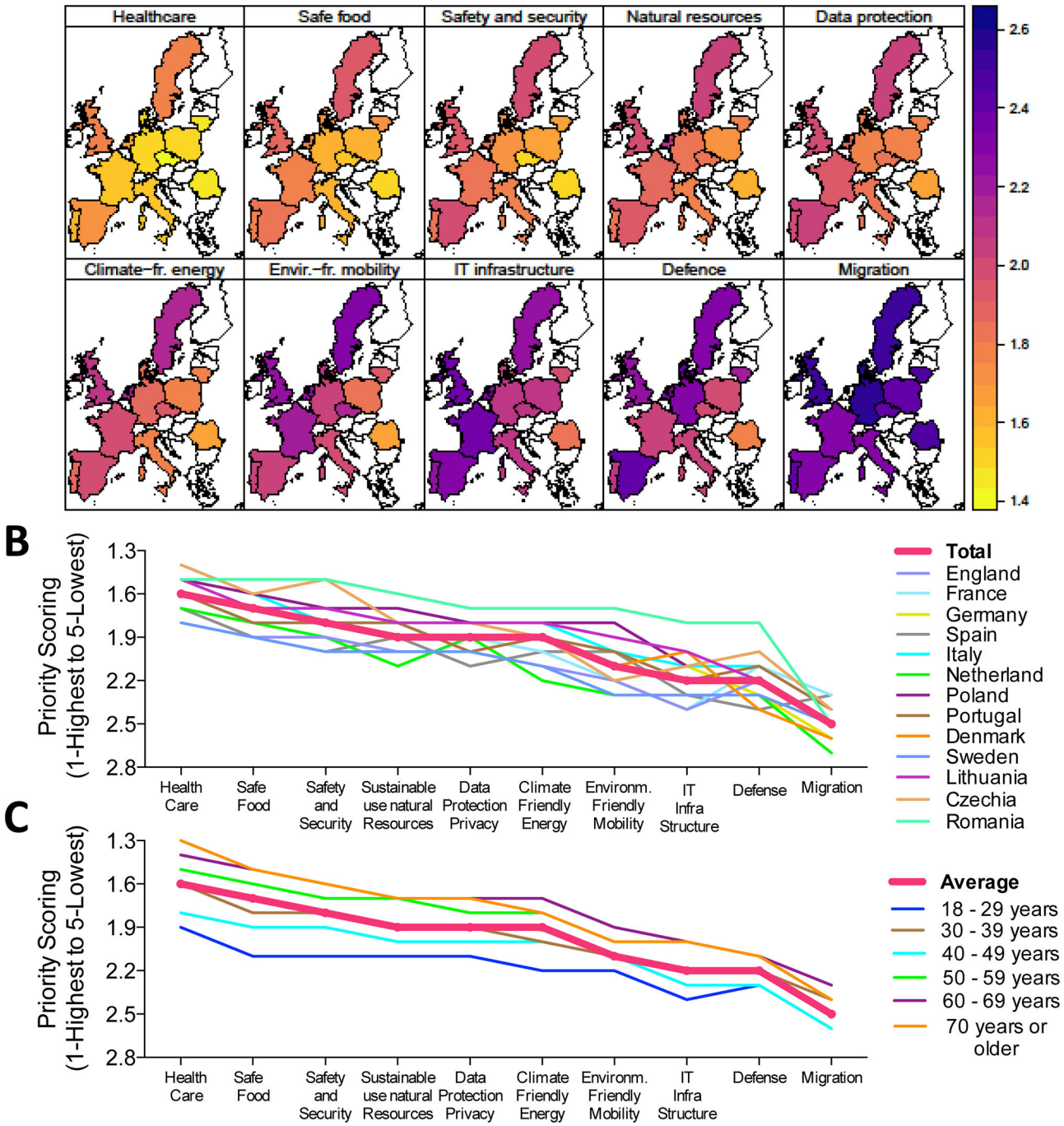


FIGURE 1 | General assessment of important social topics. Relevance of important societal topics as viewed by people in different European countries or viewed by different age groups (Scale 1—very important to 5—least important), **(A)** Geographical heat map representation of the ten studied topics, with impact scale bar shown to the right and top ranking issue 'Healthcare' depicted in yellow-orange color tones, while the lowest ranking issue 'Migration' is depicted in purple-blue color tones. **(B,C)** Numerical depiction of priority scoring sorted according to issue and country (shown in **B**) or according to issue and population age (shown in **C**).

by 35–50% of participants (lowest Germany and highest Poland, European average 45% to question A-3) (**Figure 2C**), which was considerably lower on average than the previous two questions.

Thus, on average, between 30 and 70% of respondents gave positive answers to the different introductory questions with a substantial variation between the different European

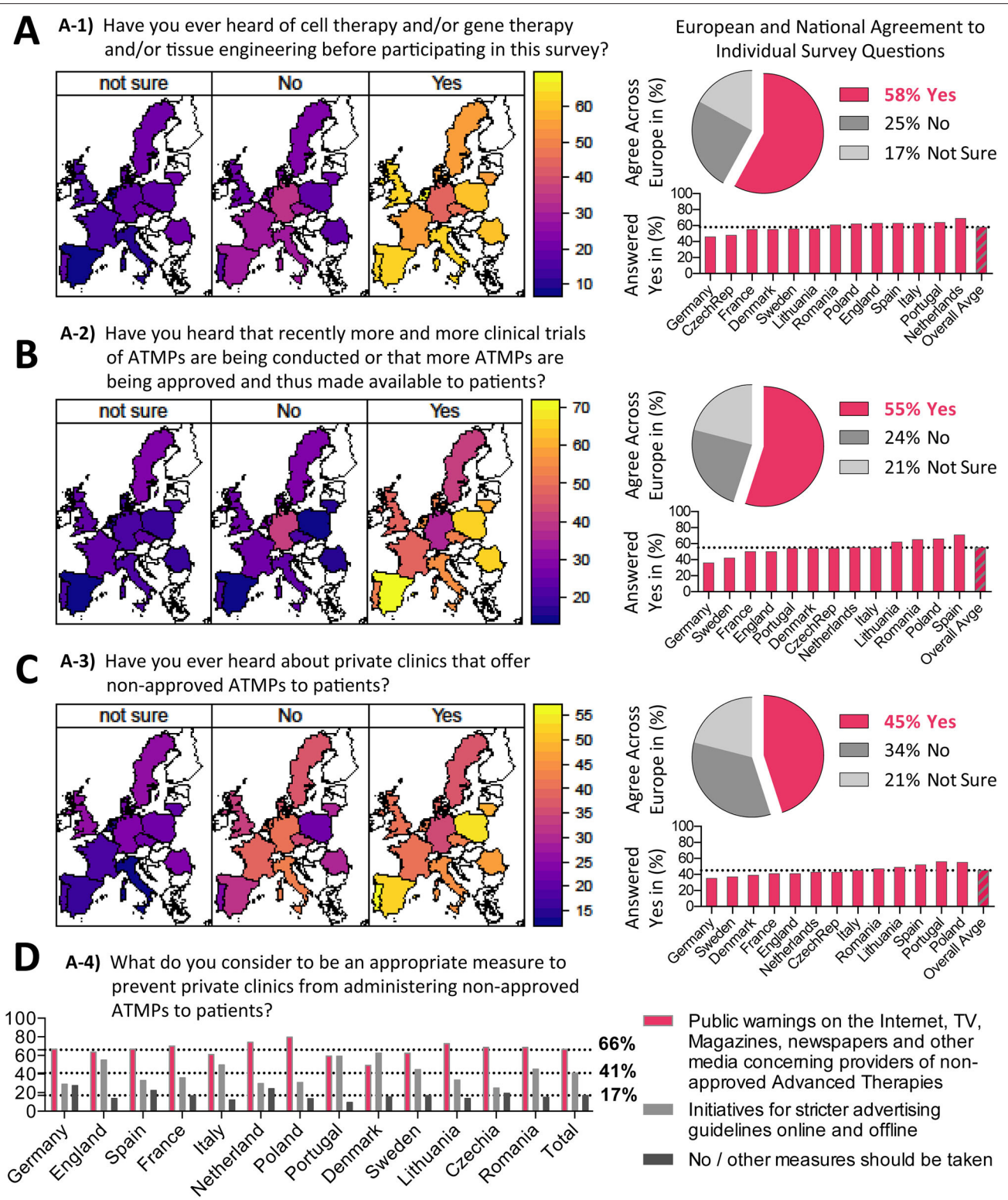


FIGURE 2 | Knowledge about ATMPs prior to survey. Results on survey topic A (see **Table 1**) sorted according to questions A1-A4. **(A–C)** Question A1-A3 geographical heatmaps shown to the left and corresponding numerical depiction shown to the right, with pie charts displaying the (%) agreement across Europe and bar charts displaying (%) answered yes per country. **(D)** Results of question A4 is displayed as bar chart (%) answered yes per country.

countries, which may be related to a combination of local presence of ATMPs and general awareness. When normalizing the data with respect to education level, again around 40–60% of European citizens gave a positive answer, thus confirming that the data are robust for differently educated people (**Supplementary Figure S2A**). A lack of knowledge on emerging new health topics could have direct consequences to public safety, which we therefore believe highlights an urgent need to improve communication to the public about ATMPs in Europe. This concerns in particular the proper use of ATMPs in a well-regulated and controlled environment vs. international medical tourism to poorly regulated regions with potential detrimental health outcomes, for example, due to a lack of sufficient quality control or its enforcement (22–24).

Next, we aimed to assess public opinion on whether this phenomenon of non-approved therapies being offered to patients should be fought, if at all, with hard measures, such as tight enforcement of the law or softer measures, such as a warning from the media. We asked in question A-4: "What do you consider to be an appropriate measure to prevent private clinics from administering non-approved ATMPs to patients" (**Figure 2D**). On average 66% of surveyed citizens supported measures such as warnings on social media, while 41% supported stricter advertising guidelines, again with considerable variation between different European countries. Our results indicate that the public is generally more supportive of soft measures to deal with this issue, while 17% of the participants voiced that no or other measures should be taken, with 7% of the participants supporting other measures (e.g., heavy fines and stricter monitoring by the health authorities), and 10% of the participants answering that no measures should be taken.

Opinions on Public Funding in Healthcare

Considering the three questions asked to EU citizens on "Public Funding in Healthcare" (**Table 1B**), the results on the first point were already outlined above in a separate section entitled "Assessment of Important Social Topics," with answers to the other two points shown in **Figure 3**. In the second question B-2, we asked: "Do you think EU- and state-funding should be invested in the development of future medical innovations?" (**Figure 3A**). An overwhelming 85% of European citizens answered with yes, ranging from 70 to 90% approval (Sweden, Denmark, and Germany lowest approval vs. Portugal, Italy, Spain highest approval, depicting a trend for a north-south divide on this issue). This question did not pertain specifically to ATMPs, but to medical innovations in general. The question served as an introduction to the following question, as it helped to change focus from the opinions of the survey participant on general medical innovations to ATMPs specifically.

The third question B-3 (**Figure 3B**): "Should EU and member states fund enabling technologies for cell and gene therapies?" found approval with 83% of European citizens, again ranging from 70 to 90% (Sweden, England, and Germany lowest approval vs. Portugal, Spain, and Italy among the highest approval, again depicting a trend for a north-south divide on this issue). Here, we asked about the support of the public for funding of R&D in technology and materials related to ATMPs. This is important, as it helps to assess whether ATMPs are medical innovations that EU citizens are interested in and thus if and how future government budgets should be allocated. Importantly, these results also held true when normalizing the data according to education level of the respondents (**Supplementary Figure S2A**). In conclusion, there appeared to be a general consensus in all countries considering the topic of funding new enabling technologies for ATMPs (Average European agreement 83–84%). Interestingly, for both questions in topic b, Southern and Eastern European countries appeared to be more supportive of investment of more public funds in healthcare. The results of the last question confirmed citizens' interest and support of public funding of missing infrastructure and technologies that could foster the development of new ATMPs.

Opinions on Reimbursement of ATMPs

Next, we assessed "Opinions on Reimbursement of ATMPs" (**Table 1C**). In the first question C-1 we asked: "Should the state pay for expensive therapies, although evidence for long-term benefit has not been shown yet?" (**Figure 4A**), to which 61% of respondents answered positively, with a considerable variation between different countries, ranging between 45 and 85% (Germany, England, and Sweden lowest approval vs. Portugal, Spain, Poland, Romania, and Lithuania highest approval). Here, it was important to us to ask a balanced question by giving an accurate description of the current scientific facts. To achieve that, we explained and emphasized the lack of long-term efficacy data for available ATMPs. ATMPs are complex products that can often only be administered by specialists in dedicated treatment centers. For rare diseases, the number of patients is often low and considering financial feasibility it is not possible to open dedicated treatment centers in every region or country. Statutory coverage of cross-border healthcare would mean the taxpayer funds treatments given in another European country. Thus, we aimed to find out if European citizen support this reimbursement concept. We asked: "Do you agree, that in the case of rare disease, cross-border health care (e.g., traveling abroad) is the best way to provide the most beneficial treatment for patients?" (**Figure 4B**), which 74% of respondents answered positively, again with a quite substantial variation between different EU nations, ranging between 60 and 80% (Sweden, England, Germany, and France lowest approval vs. Portugal, Romania, Lithuania, and Poland highest approval). In the last question we asked: "Should non-medical costs be covered in cross-border healthcare?" (**Figure 4C**), which 70% of European citizens approved of, once more with a large variation between countries, ranging between 50 and 80% (England, Sweden, Denmark, and Germany lowest approval vs. Portugal, Spain, Italy, as well as Lithuania and Romania highest approval). This question pertained to one of the big hurdles in reimbursement of cross-border healthcare: "If medical treatment is administered abroad, the non-medical costs

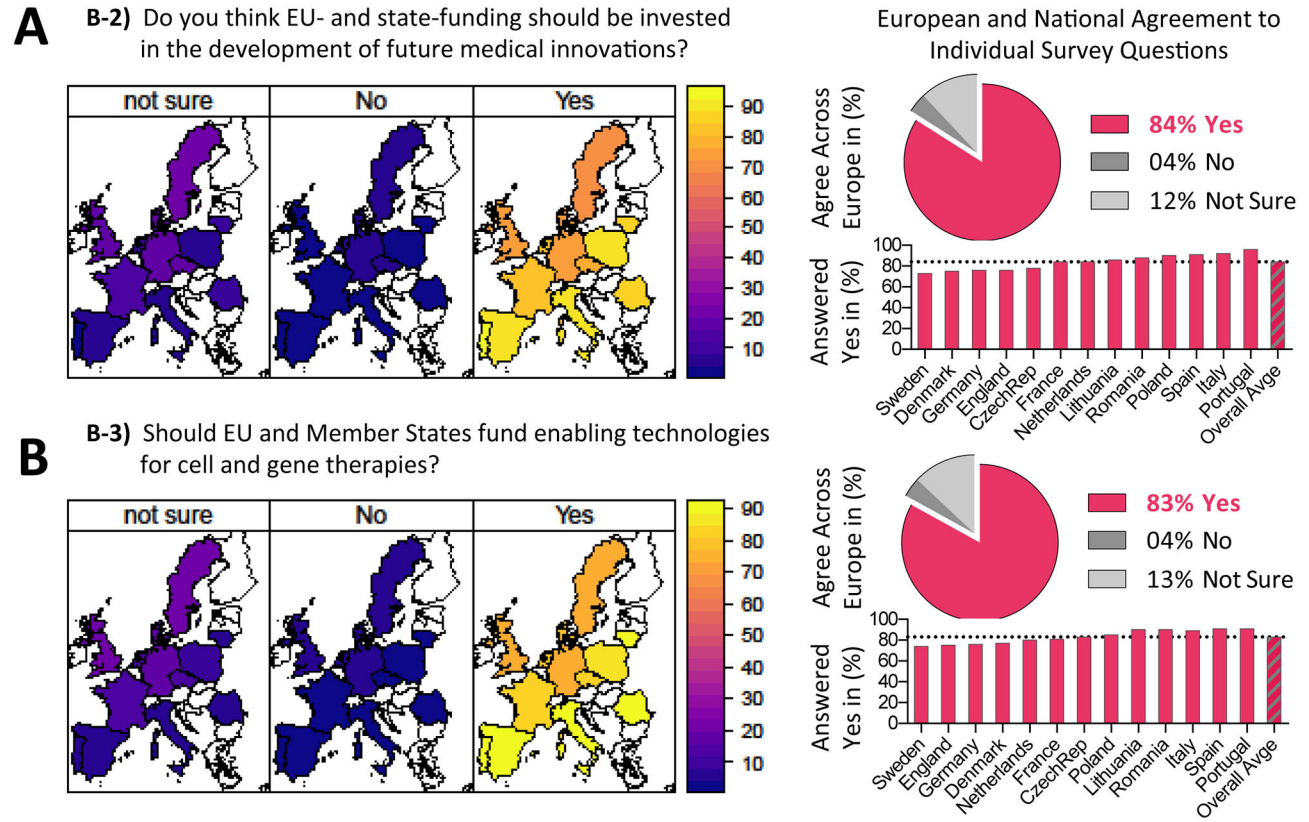


FIGURE 3 | Opinions on public funding in healthcare. **(A,B)** Results on survey topic B sorted according to questions B2-B3 (Question B1 is show separately as introductory topic in **Figure 1**) with geographical heatmaps shown to the left and corresponding numerical depiction shown to the right, with pie charts displaying the (%) agreement across Europe and bar charts displaying (%) answered yes per country. Interestingly, an overwhelming 84% of European citizens agree that EU- and state-funding should be invested in the development of future medical innovations, while 83% agree that EU and member states should fund enabling technologies for cell and gene therapies, with only 4% of EU citizens answering 'No' and 12-13% answering 'Not Sure', indicating a strong support of EU citizens for funding future medical innovations and enabling technologies for cell and gene therapies.

(e.g., cost of travel and accommodation) are not usually covered by health insurers." We were interested in knowing the opinion of the public on the possibility of reimbursing non-medical costs, by law, in case of cross-border treatment. Again, the opinions of European citizens on reimbursement also held true when normalized for Education Level (**Supplementary Figure S2C**). In conclusion, our results demonstrate the European public's general acceptance of high prices with only 11% of the people clearly objecting to this policy (**Figure 4A**). Important for future discussions was the finding that there was strong support for other aspects of reimbursement, such as the concept of cross-border healthcare and the aspect of reimbursement of non-medical costs (**Figures 4B,C**).

To lead over to the discussion of the data resulting from this survey, we prepared a graph entitled: "Relationships Between Various Themes and How They Affect the Overall Acceptance of Cell and Gene Therapies" (**Figure 5**), which was drafted according to a prior design by Aiyegbusi et al. first presented in their review "Patient and Public Perspectives on Cell and Gene Therapies" (21). This figure elegantly illustrates the interrelationship of the different themes.

DISCUSSION

Recent advances in the field of Advanced Therapies and ATMPs have triggered great interest and responses from the scientific community, the healthcare sector, politicians, and other professionals. Our survey aimed to give an update on the public view on matters related to ATMPs particularly in Europe, as one of the biggest healthcare markets with around 500 million citizens. The survey is also of interest from the perspective of resource allocation through public funding. If one follows the media reports, especially before the Corona pandemic, topics such as environmental protection and migration appeared to dominate the public interest. However, our survey reflects a different narrative. Considering the knowledge the public has on the subject of ATMPs based on the survey results, we can conclude that the new advances in these therapies have not escaped public attention. Indeed, the part of the survey pertaining to public's knowledge about ATMPs could serve as a standard for future studies to measure improvements in knowledge and awareness of ATMPs by the layperson. Our results demonstrate that healthcare is by far one of the most important social topics

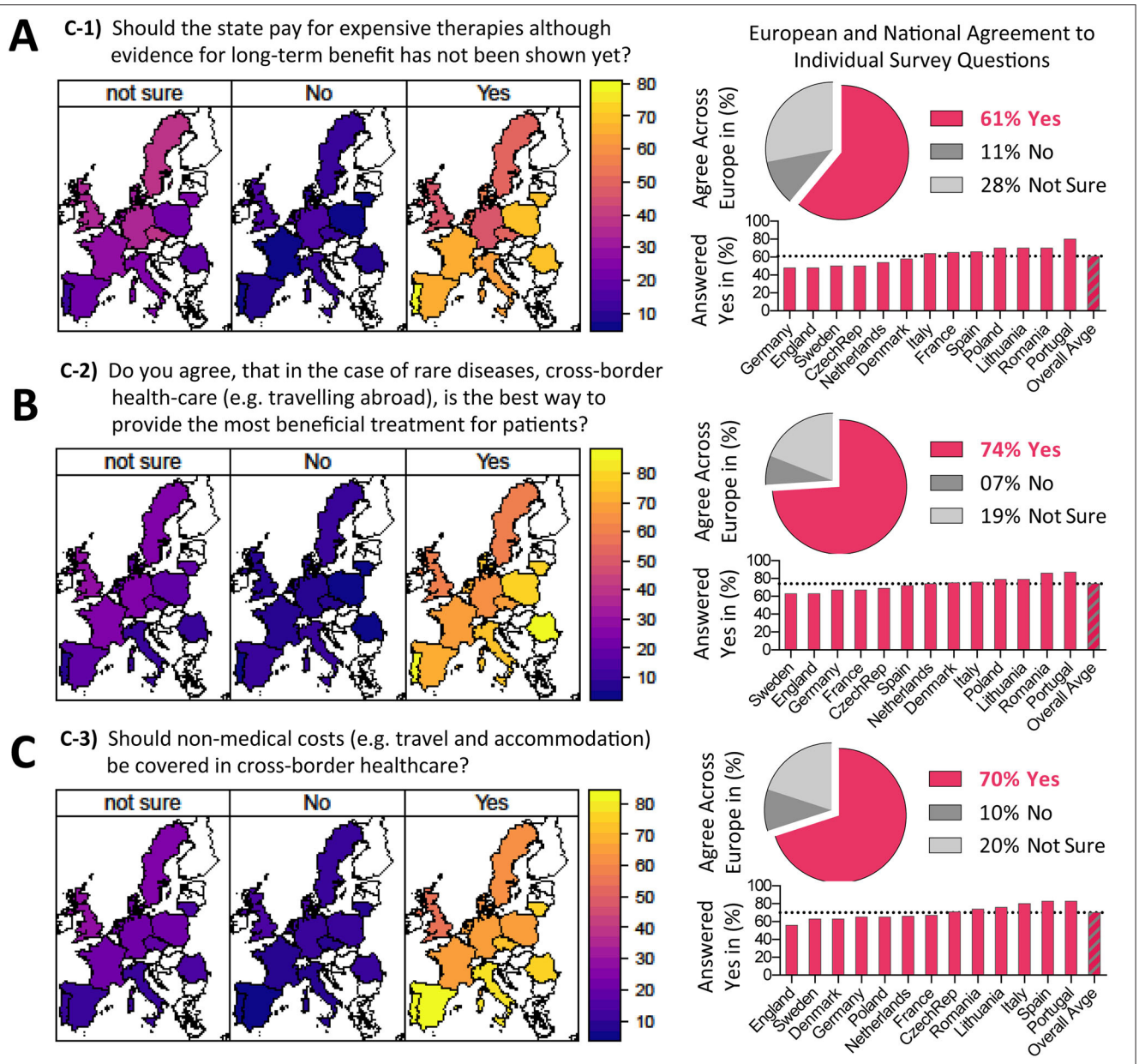


FIGURE 4 | Opinions on reimbursement of ATMPs. **(A–C)** Results on survey topic C sorted according to questions C1-C3, with geographical heatmaps shown to the left and corresponding numerical depiction shown to the right, with pie charts displaying the (%) agreement across Europe and bar charts displaying (%) answered yes per country. Only 61% of European citizens agree that the state should pay for expensive therapies although evidence for long-term benefit has not been shown yet, while 74% agree that in the case of rare diseases cross-border health-care (e.g. traveling abroad) is the best way to provide the most beneficial treatment for patients, and 70% agree that non-medical costs (e.g. travel and accommodation) should be covered in cross-border healthcare, clearly indicating that a majority of European citizens is in support of European cross-boarder health care for rare diseases and for support for non-medical costs, such as travel and accommodation.

for European citizens in all of the surveyed countries and further, that they are extremely supportive of public funding in healthcare innovations. Moreover, the public is supportive of investing in infrastructure and enabling technologies that may lead to the development and market introduction of more ATMPs. The survey shows that it is the will of the surveyed European population that EU and state-funds should be used to support ATMPs. Further targeted allocations of EU funding for ATMPs should thus be made since it reflects the explicit interests of the European people.

The scale of cost for well-funded and staffed healthcare systems, e.g., in context of the development of new therapeutic options such as ATMPs and their adjunct infrastructure, may be viewed critically in different contexts, given the limited national budgets. On the one hand, investment into new technologies may be perceived as competition to the standard-of-care, on the other

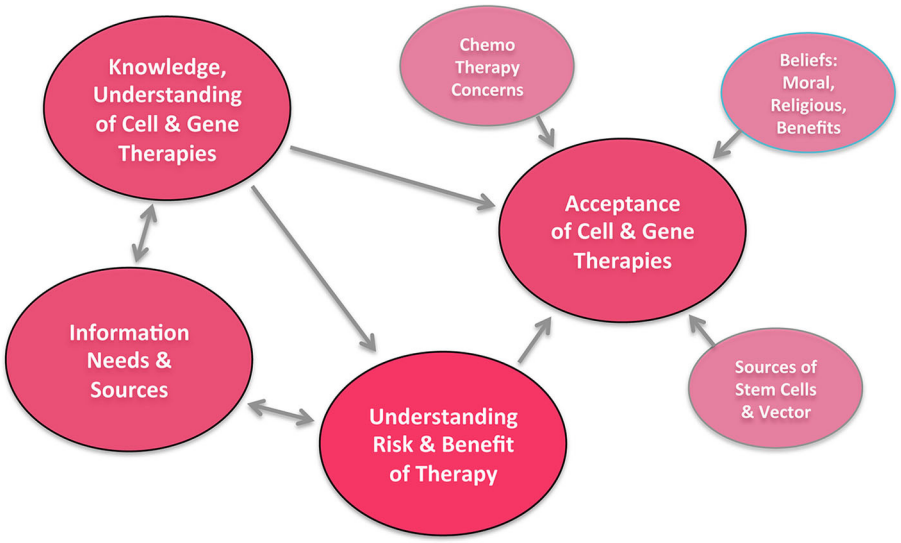


FIGURE 5 | Relationship between various themes and how they affect overall acceptance of cell and gene therapies, adapted from Aiyegbusi et al. (21). In our "ATMP EU Survey," the highlighted four main groups (emphasized larger circles) were found to be of key importance.

hand, new therapeutic options may also provide both financial and medical long-term benefit in changing demographics. There are always limitations to expensive therapies, especially in providing prolonged symptomatic, but not curative treatments, which must indeed be seen in the context of society as a whole. Indeed, overall cost-effectiveness has actually been one of the driving forces behind ATMP development, to offer novel, better, more sustainable, and in the end also cheaper cures, to eventually improve the quality of live for patients and their ability to actively participate as healthy independent and productive subjects of society. Novel ATMPs may often be perceived as costly treatments. However, as we have already outlined in our introduction, ATMPs can be developed for both, common pathologies, but also for very specific indications with (so far) unmet medical need, provided the clinical benefit, price and regulatory requirements warrant the effort. Thus, ATMPs may range from cost-efficient and broadly marketed "off-the-shelf" therapeutics for common pathologies, to highly specific, but in the case potentially more costly treatments, to target otherwise difficult or impossible to treat indications. Importantly, although this point may be subject to global regional differences, at least in the EU market area it may not be the primary goal to develop "Luxury ATMPs for wealthy clients with deep pockets," but to provide novel, cost-efficient/competitive, and more sustainable treatments. We are much aware that some ATMP-developments for yet unmet medical needs can be costly in certain indications, e.g., when aiming to provide live-saving treatments for otherwise incurable disease, which is accompanied by a hot debate about proper reimbursement strategies for such cases. In addition, the recent Covid-19 pandemic has clearly illustrated the value of a highly adaptive medical/research infrastructure. Overall, we do not expect that ATMPs would diminish the standard of care in Europe by driving out investment in other healthcare areas, but rather that they enrich the therapeutic spectrum/options, with the prospects of adequate long-term benefit still needing to be evaluated in the future.

Indeed, the transformative or even disruptive potential of ATMPs to change existing modes of healthcare has been communicated to the public for many decades (25). While the concept has always been straightforward—replace and correct disease-causing faulty genes, harness regenerative properties of human cells for the treatment of diseases or to repair defective tissues—the actual implementation of these concepts has experienced ups and downs, with many candidates failing due to ineffectiveness, as well as safety and quality issues (10–12, 24). Importantly, some approved products were even withdrawn due to lack of commercial success (5, 16, 26, 27). In the recent years we have witnessed new ATMPs with unprecedented therapeutic efficacy that have already reached the market, such as gene therapy for rare diseases and CAR T-cell therapies for hematological cancers (16, 18, 19). These new medical breakthroughs have made it clear that the potential can be translated into therapeutics and that the transformative promise can be made reality. As a result, the budget invested in research and development of ATMPs has increased rapidly, evident by the increasing number of biotech startups in the field, as well as the attention and resource allocation in more established pharmaceutical companies. Benefit to patients has been observed in an increasing number of both clinical trials and expected regulatory approvals of ATMPs on the market. However, the assessment of ATMPs has been troublesome for regulators. Manual manufacturing with new manufacturing technologies, complex raw materials, difficult-to-characterize products and difficulties in the design of clinical trials have been a hurdle for both developers and regulators assessing them. In addition, the workload of regulators has increased significantly due to

an influx of new applications whilst recruitment of suitable technology specialists in the fields has become increasingly more challenging due to competition with industry in the same limited pool of scientists. In addition, ATMPs have also been challenging for health insurers and policy makers. For example, how to pay for ATMPs that (unlike traditional medications) are ideally given only once but can be very expensive, as well as ensuring patients access to treatment in specialized centers. Here, policy makers are entrusted with the task of encouraging research and development, and being able to identify and tackle roadblocks present with the emergence of this novel field.

Without doubt, there is a notable gap between the public awareness of the existence of ATMPs and the awareness of unapproved ATMPs offered by private clinics in both developed and developing countries. In particular, this concerns the proper use of ATMPs in a well-regulated and controlled environment vs. international medical tourism to poorly regulated regions, which may not be in the interest of European Health Care Policy. These therapies may not only lack evidence for efficacy and sufficient quality control, but can even have potential detrimental health outcomes (22–24). There is a notable trend is flourishing of private clinics offering unapproved ATMP for treatment of a range of medical conditions, from orthopedic problems to cancer, autism and even COVID-19 (11, 22–24, 28). Due to the potential risk of unapproved ATMPs, we conclude that there is room for improvement in communication and explanation to the public. This path of action seems to be preferable because it is widely supported by the public and because direct legal measures against such offerings do not seem to be a very effective tool for this purpose. In summary, this new class of medicines poses challenges for every stakeholder in the healthcare sector. Knowledge of public perceptions is needed so that it can help guide efforts to "educate the public" (29). A paper from Robillard et al. shows that public perceptions and therefore trust in emerging biotechnologies are important for the research process, specifically, through channels such as funding and public advocacy (30). In a recent paper, 1,561 articles examining opinions and attitudes on gene therapies were systematically reviewed (31). After review, 41 articles and their results were included in the study. The most relevant points for this paper are the following: Somatic therapies had higher levels of acceptability than germ line therapies, public acceptance of treatments is essential for future clinical trials, and clinicians and scientists must be clear and open with the public about the risks and benefits while also encouraging further education of individuals not naturally interested in science.

Aiyegbusi et al. provide further insights into the public perception of gene and cell therapies with their systematic review of 10,735 papers (21), which were then narrowed down and a total of 33 were selected for full review. Their review found that patients desire more information regarding cell and gene therapy treatments, regardless of age, gender, and education. They found that acceptance of these therapies increased with the dispersion of information, and that patients tend to overestimate the benefits and underestimate the risks of ATMPs, probably simply due to their underreporting (23, 24). **Figure 5** represents the relationships between various themes and how they affect overall acceptance of cell and gene therapies. Our survey presented here portrays the current perception of the European citizens, aiming at identify and categorize their priorities when it comes to decisions on spending and funding for research and development. Potential next steps for future research are identifying why European Citizens prioritize the policies focused on in this paper, and perhaps more importantly, if individuals are interested in greater spending on translational research vs. traditional basic research. Many studies regarding public opinions, beliefs, and perception of ATMPs concern gene therapies. Historically, a technological milestone in the advancement of gene therapies was achieved in 1990, when the first therapeutic gene transfer in adenosine-deaminase-deficiency (ADA) patients was carried out, evoking a strong increase in the public's interest. In 1992, Macer et al. found that 54% of the Japanese public were in favor of gene therapies (32). It is important to note that the paper's phrasing of gene therapy questions emphasized the person's opinion in a life-threatening situation, such as a fatal disease. In 1993, the same authors broadened their research and focused on an additional six countries, Australia, India, Israel, Japan, New Zealand, Russia, and Thailand (33). Here, ∼75% of respondents supported the personal use of gene therapies. However, 1999 brought negative press to gene therapies with the death of Jesse Gelsinger the first person publicly identified as having died in a clinical trial for gene therapies. Interestingly, in the year 2000, Gaskel et al. published that public respondents were much more in favor of the application of biotech research to medicine and the environment than they were of its application to food (34).

Alison Abbot et al. highlighted that the focus of gene therapists from the early 1990s has transitioned from completely fixing damaged genes to now treating conditions (35). In a sense, gene therapists have become noticeably more realistic with their goals. In 2002, Gottweis et al. provided a fascinating and deep analysis of public perception of gene therapy (29). Although public attitude toward innovation in general and more specifically biotechnology may have changed significantly since then, the main point by Gottweis may still be valid: The understanding of the science behind gene-therapy plays a smaller role than the trust in scientific institutions when it comes to public perception. In other words, attitudes toward gene-therapy are more related to trust than to knowledge. In 2003, China became the first country to approve a gene therapy-based product for clinical use. In 2008, the first phase III gene therapy clinical trial was successfully completed in the EU. Three years later, the European Medicines Agency recommended for the first time a gene therapy product for approval in the EU (36). Generally, the public has had very positive attitudes toward biotechnology applications in the health-care sector, especially, when these applications seek to treat severe diseases. Importantly, given the promise of ATMPs as life-saving cures, pricing and reimbursement models of ATMPs, with their long lasting treatment effects after one application, are expected to be quite different from those of traditional pharmaceutical products given on a daily or a weekly basis. Additionally, patient access to highly complex medicinal products, that often require highly specialized treatment centers, are expected to be

different than access to traditional pharmaceutical products, that can usually be administered in many centers or clinics. This is especially an issue in the case of rare diseases, where the number of patients may not be enough to justify setting up a highly specialized treatment center in every country. In this case, a cross-border approach may be the right solution. Our data shows that the EU citizens are generally open to paying higher prices for ATMPs with potentially long-lasting effects and furthermore that they are open to reimbursement models for cross-border healthcare.

CONCLUSIONS AND LIMITATIONS

Considering limitations, one must first of all acknowledge that the people who decided to participate in this survey were most likely generally interested in the survey's topic and therefore the survey may be biased in the positive direction. This is often the case when conducting surveys, since the participant is first asked whether she/he would like to participate in a survey about a specific topic. Another important point, although the population in the countries surveyed in the study amount to roughly 85% of the population in Europe, the survey only included 13 out of 28 countries in Europe (including the UK). However, we surveyed countries from all geographic regions in the EU and in different economic situations. Lastly, it was our deliberate choice to have the same weight for each country, although their population sizes vary greatly. Many of the questions asked were about scientific matters and complex concepts. Ideally, every question would have followed a lengthy explanation of the background to it. However, we were limited in the time the survey consumes and therefore in the length and complexity of the explanations preceding each question. This may have resulted in misinterpretation of some of the questions. Overall, we found in this survey that more than 80% of the participants supported public funding for general medical innovations and more than 80% of the participants supported public funding for the development of better and more efficient materials and technology specifically in the ATMP field, indicating great public interest. Sixty-one percentage of participants supported statutory reimbursement for very expensive ATMP treatments despite the fact that the effectiveness of many of these therapies has only been demonstrated over a short time period, and information on their long-term benefits are currently lacking. Furthermore, when presented with the problem of complex treatments for rare diseases, which can involve treatment abroad, 74% of the participants supported the model of cross-border healthcare in specialized treatment centers. Again, suggesting that there is public support for state funding of ATMPs, including coverage of medical and non-medical costs in other EU countries. We therefore believe the results of this survey, representing the views across a range of European countries and citizens, demonstrate a clear indication for national and EU funding bodies to invest in healthcare and the future of healthcare. ATMPs hold great promise and potential to revolutionize this field for the benefit of European society if sufficient time and investments are made now.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GG, CH, SS, NB, FS, PR, ZA, RO, GD, SB, NN, RR, GM, and HDV contributed to conception and design of the study. GG and CH organized the database. GG, CH, SS, and GM performed the statistical analysis. GG, CH, GM, and HDV wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was funded by the European Union's Horizon 2020 research and innovation program under the Grant Agreement No. 820292 (RESTORE; https://www.restore-horizon.eu/) and in part also by Projects No. 733006 (PACE) and No. 779293 (HIPGEN). This study was also made possible by funding through the German Research Foundation (DFG) and German Federal Ministry of Education and Research (BMBF) through BSRT (GSC203) and BCRT. We acknowledge support from the DFG and the Open Access Publication Fund of Charité – Universitätsmedizin Berlin.

ACKNOWLEDGMENTS

We would like the thank Cecilie Löe Licht, Joana Namorado, and Diana Steffen for reviewing the questions and helping with translations to other languages, and Francesco Grassiccia from Innovation Acta S.r.l. for support with the graphical depictions. Furthermore, we would like to thank the following members of the RESTORE Consortium for their support with this project: Maximilian Emmert and Simon P. Hoerstrup from University of Zuerich, Switzerland; Michaela Sharpe and Terri Gaskell from Cell and Gene Therapy Catapult, London, UK; Silke Hoffmann and Uwe Marx from TissUse GmbH, Berlin, Germany; and Michael Apel and Mario Assenmacher from Miltenyi Biotech GmbH, Bergisch Gladbach, Germany.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.739987/full#supplementary-material

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Conflict of Interest: ZA and RO are employed by Pluristem Therapeutics Inc. Haifa, Israel. GD is employed by Innovation Acta S.r.l., Italy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 03 November 2021
doi: 10.3389/fmed.2021.742436

Novel Targets in a High-Altitude Pulmonary Hypertension Rat Model Based on RNA-seq and Proteomics

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High-altitude pulmonary hypertension (HAPH) is a complication arising from an inability to acclimatize to high altitude and is associated with high morbidity and mortality. We aimed to analyze the effects of macitentan, selexipag, riociguat, and reoxygenation on HAPH, and to screen possible targets of these treatments for future drug screening. Rats were subjected to hypobaric hypoxia for 35 days to induce HAPH, and treated with vehicle or selexipag, macitentan, riociguat, or with reoxygenation, from days 21 to 35. Selexipag, macitentan, and reoxygenation prevented an increase in mean pulmonary artery pressure and hypoxia-induced right ventricular hypertrophy, compared to the vehicle. Riociguat had little effect. RNA-seq and proteomics revealed strong correlations between responses to the three drugs, which had almost identical effects. GO-enrichment revealed that the differentially expressed genes included those involved in metabolic regulation, transcription, and translation. Various molecular pathways were annotated. Selexipag, macitentan, and reoxygenation ameliorated HAPH. Serpina1, Cryz, and Cmc1 were identified, *via* multi-omics screening, as key genes involved in HAPH. These findings provide new insights into the targeted drug mechanisms in HAPH.

Keywords: high-altitude pulmonary hypertension, selexipag, macitentan, RNA-seq, proteomics, reoxygenation

OPEN ACCESS

Edited by:
Hans-Dieter Volk,
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Reviewed by:
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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *16 July 2021*
Accepted: *11 October 2021*
Published: *03 November 2021*

Citation:
Xu X, Li H, Wei Q, Li X, Shen Y, Guo G, Chen Y, He K and Liu C (2021) Novel Targets in a High-Altitude Pulmonary Hypertension Rat Model Based on RNA-seq and Proteomics. Front. Med. 8:742436. doi: 10.3389/fmed.2021.742436

INTRODUCTION

Pulmonary hypertension (PH) refers to a resting mean pulmonary artery pressure ≥25 mmHg. It is divided into five categories, based on hemodynamic characteristics, pathogenesis, and pathology (1). High-altitude pulmonary hypertension (HAPH), in the third category, is caused by a compensatory increase in lung ventilation and pulmonary arteriole vasoconstriction in a high-altitude hypoxic environment and leads to high altitude-induced cardiomyopathy. The clinical symptoms include exercise dyspnea, headache, and fatigue. HAPH may reflect a failure to acclimatize to high altitude, accompanied by unclear pathophysiological mechanisms. About 140 million people live at >2,500 m above sea level, and >40 million people visit these high-altitude regions each year (2, 3). Hypoxia causes the gene expression profiles of organs to change differentially; this is known from adaptive changes in the genotype of long-term high-altitude residents and their offspring (4).

Treatments for HAPH are still being investigated. Patients should be advised to move to lower altitude, and oxygen therapy is effective (5). Studies of drug treatment are limited to some randomized trials (6). HAPH has similar pathology to other types of PH, so it is worth

considering whether drugs that are effective for PAH might be effective for HAPH. In addition to conventional drugs, such as vasodilators, targeted drugs have been increasingly studied. Activation of endothelin receptor 1 leads to pulmonary vasoconstriction and smooth-muscle cell proliferation. Macitentan is a dual antagonist of the endothelin receptor, with enhanced penetration and low risk of hepatotoxicity when administered for pulmonary arterial hypertension (7–10). Prostacyclin is released by endothelial cells and promotes pulmonary vasodilation, with antithrombotic and antiproliferative effects. Selexipag, an oral prostacyclin (PGl2) receptor agonist, differing in structure from prostacyclin, has been reported to be more effective than placebo in reducing morbidity and mortality in patients with PAH (11, 12). Endothelial nitric oxide (NO) production was lower and phosphodiesterase type 5 expression was higher in pulmonary artery smooth-muscle cells and in the right ventricular myocardium in patients with PAH than in those without PH (13–15). NO activates soluble guanylate cyclase (sGC), stimulating cyclic guanosine monophosphate (cGMP) production, leading to vasodilation of small arteries, and inhibiting cell proliferation. Further, phosphodiesterase type 5 hydrolyzes cGMP. Riociguat is a soluble guanylate cyclase stimulator, and can promote vascular remodeling and pulmonary vasodilation without depending on NO. Riociguat increases the sensitivity of sGC to NO, thus raising cGMP levels (16, 17).

However, all of these targeted drugs have been studied in patients with symptomatic PH, whose pathogenesis was idiopathic, familial, and was associated with connective-tissue disease, portal hypertension with liver cirrhosis, or toxin exposure. Almost no HAPH patients have been included in these studies. Therefore, the effectiveness and mechanisms of these drugs in HAPH remain unclear. To address this, we aimed to examine the molecular mechanisms involved in hypoxia, using a rat HAPH model, and applying an integrated multi-omics approach. In doing so, we aimed to investigate the effects of several targeted drugs and reoxygenation to identify potential new therapeutic targets for altitude-induced hypoxia. In particular, we examined how the targeted drugs affected differential gene and protein expression among organs.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (male, 220–250 g) were purchased from the Animal Experiment Center of the Chinese PLA General Hospital (Beijing, China). All animal experimental procedures were approved by the Animal Ethics Committee of the Chinese PLA General Hospital (approval number: 2017-X13-05). The animals were raised under a 12 h light/dark cycle, with free access to food and water. Room temperature was 22–25°C. Bedding was changed twice a week. Control group rats were housed in a normoxia environment and treated with vehicle after 3 weeks. The remaining animals were randomly assigned to six groups at 3-week time point, one group was sacrificed for assessing the cardiopulmonary function on day 21, the other five groups were M (model group): chronic hypoxia, vehicle-treated; PGI2Y: selexipag-treated (5 mg/kg, bid.); ETAY:macitentan-treated (30 mg/kg, q.d.); SGCY: riociguat-treated (10 mg/kg, q.d.); and RE: reoxygenation-treated. Except for group C, the other five group rats were housed in a hypobaric hypoxia chamber for 35 d; treatments started on day 21 and continued for 2 weeks. Each group had 6 rats. We regarded the reoxygenated group as the positive control group. Selexipag, macitentan, and riociguat were purchased from Selleck Chemicals (catalog numbers S3726, S8051, and S8135; USA).

Chronic Hypoxia-Induced PH and Drug Treatment

The rats were placed in a 10% O_2 (hypoxic) chamber for 3 weeks to develop PH. Control rats (group C) were housed under normoxia for the entire experiment and treated with the vehicle during the treatment period (i.e., from day 21). At 3 weeks, the remaining rats were randomized into six groups; one group was sacrificed (day 21) to assess cardiopulmonary function. For euthanasia, animals were anesthetized by pentobarbital sodium (90 mg/kg body weight), and mean pulmonary arterial pressure (mPAP) was calculated to validate the model. The remaining five groups were treated with targeted drugs or reoxygenation, from day 21 to sacrifice on day 35. These groups were; chronic hypoxia, vehicle-treated (M); chronic hypoxia, selexipag-treated (5 mg/kg, bid.) (PGI2Y); chronic hypoxia, macitentan-treated (30 mg/kg, q.d.) (ETAY); chronic hypoxia, riociguat-treated (10 mg/kg, q.d.) (SGCY); chronic hypoxia, reoxygenation (RE). Selexipag, macitentan, and riociguat were purchased from Selleck Chemicals (catalog numbers S3726, S8051, and S8135; USA).

Hemodynamic Measurements and Sample Collection

Vascular pressure was assessed using Millar catheters, as previously described (18). Rats were fixed on the operating table, anesthetized, tracheotomized, and placed on ventilator-assisted breathing (Kent Scientific, USA). A Millar SPR 838 pressure–volume catheter (ADInstruments, USA) was inserted through a parasternal incision into the right ventricle (RV), then advanced into the pulmonary artery. Pressure measurements were acquired using an MPVS Ultra system coupled to a PowerLab data acquisition system (ADInstruments) to calculate mPAP.

Sample Preparation and Assessment of RV Hypertrophy

After catheterization and measurements, the lungs and heart were harvested, and washed twice with ice-cold saline to remove

Abbreviations: HAPH, high-altitude pulmonary hypertension; NO, nitric oxide; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate; PGI2Y, selexipag-treated group; ETAY, macitentan-treated group; SGCY, riociguat-treated group; RE, reoxygenation group; LV, left ventricle; RV, right ventricle; mPAP, mean pulmonary arterial pressure; WGCNA, Weighted Gene Co-expression Network Analysis; TOM, topological overlap matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DTT, dithiothreitol; IAA, iodoacetamide; iBAQ, intensity-based absolute quantification; AUC, area under the curve; FOT, fraction of total; IL-1, interleukin-1; TNF-a, necrosis factor a; COPD, chronic obstructive pulmonary disease; GLS, glutaminase.

blood and other contaminants. The RV and left ventricle (LV) with septum (LV + IVS) were weighed, and their mass ratio, the RV hypertrophy index, RVHI = RV/(LV + IVS), was calculated. The upper left lung was fixed by inflation with 10% formalin, embedded in paraffin, and sectioned for histology.

RNA-seq Analysis

RNA Extraction and Qualification

RNA from the samples was extracted using TRIZOL (1 mL/200 mg, Life Technologies, Rockville, MD) according to the manufacturer's protocol. RNA quality and concentration were checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Purified RNA was stored at −80°C until required. RNA samples were reverse-transcribed using the Reverse Transcription System (Promega, USA).

RNA-seq and Computational Analysis

Total RNA was extracted, and mRNA and noncoding RNAs were enriched by depleting rRNA using an Arraystar rRNA Removal Kit. The mRNAs and noncoding RNAs were broken into short fragments (∼200–500 nt) by the fragmentation buffer. The short fragments were used as templates, and first-strand cDNA was synthesized using random hexamer primers. dTTP was replaced by dUTP during second-strand synthesis. Elution buffer was then added to purify and resolve the short fragments *via* end-repair and addition of adenine. Short fragments were purified and connected with adaptors; the second strand was digested using uracil-N-glycosylase (19). After agarose gel electrophoresis, suitable fragments were selected as templates for PCR amplification. Quantification and quality assessment of the sample library were performed using an Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System. The library was sequenced using an Illumina HiSeq 2000 system.

Protein concentration was determined *via* Bradford Protein Assay. The samples were analyzed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) interfaced with an Easy-nLC 1000 nanoflow LC system (Thermo Fisher Scientific). For each sample, 4 μL of digested protein was loaded onto a Biosphere C18 Precolumn (2 cm × 100 μm; particle size, 3 μm; pore size, 300 Å) at 7.5 μL/min. After 3 min, the protein samples were separated using a 150 μm × 12 cm silica microcolumn (homemade; particle size, 1.9 μm; pore size, 120 Å) with a linear gradient of 5–35% mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 600 nL/min for 75 min. Using a data-dependent strategy, by measuring MS1 in an Orbitrap mass spectrometer with a resolution of 120,000, and then using high-energy collision dissociation with a normalized collision energy of 27% and a dynamic rejection time of 18 s, the first 20 precursors were subjected to tandem mass spectrometry. Trypsin digestion of 293T cells was used for routine quality control of samples, to ensure sensitivity and repeatability.

Data Preprocessing

Transcripts with fragments per kilobase of transcript per million mapped reads (FPKM) >0.1 in at least 20% of the samples were retained for NCBI omics-database screening. Sequence expression was subsequently normalized using quantiles (20). For the logistic regression analysis, feature expression was further normalized to a normal distribution using the z-score algorithm (21).

Proteomic Measurement

Protein Trypsin Digestion

Whole-tissue protein extractions: 0.1 g tissues were lysed with 400 μl urea lysis buffer (8 M urea, 100 mM Tris-HCl pH 8.0), 4 μl protease inhibitor (PierceTM, Thermo Fisher Scientific) was added to protect protein from degradation and protein concentrations were measured using Bradford method (Eppendorf Biospectrometer). One hundred micrograms of proteins were digested by FASP procedure. Namely, the protein samples were supplemented with 1 M dithiothreitol (DTT) to a final concentration of 5 mM and incubated for 30min at 56°C, then added iodoacetamide (IAA) to a 20 mM final concentration, and incubated in the dark at room temperature. After half an hour incubation, samples were added 5 mM final concentration of DTT and keep in dark for another 15 min. After these procedures, protein samples were loaded into 10 kD Microcon filtration devices (Millipore) and centrifuged at 12,000 g for 20 min and washed twice with Urea lysis buffer (8 M Urea, 100 mM Tris-HCl pH8.0), twice with 50 mM NH_4HCO_3. Then the samples were digested using trypsin at an enzyme to protein mass ratio of 1:25 overnight at 37°C. Peptides were extracted and dried (SpeedVac, Eppendorf).

LC-MS/MS Analysis

Samples were analyzed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Rockford, IL, USA) connected to an Easy-nLC 1000 liquid chromatography system (Thermo Fisher Scientific). Dried peptide samples were re-dissolved in Solvent A (0.1% formic acid in water) and loaded to a trap column (100 μm × 2 cm, homemade; particle size, 3 μm; pore size, 120 Å; SunChrom, USA) with a max pressure of 280 bar using Solvent A, then separated on a home-made 150 μm × 30 cm silica microcolumn (particle size, 1.9 μm; pore size, 120 Å; Dr. Maisch GmbH) with a gradient of 5–35% mobile phase B (acetonitrile and 0.1% formic acid) at a flow rate of 600 nl/min for 150 min. The MS analysis for QE HF was performed with one full scan (300–1,400 m/z, R = 120,000 at 200 m/z) at automatic gain control target of 3e6 ions, followed by up to 30 data-dependent MS/MS scans with higher-energy collision dissociation (target 2e4 ions, max injection time 40 ms, isolation window 1.6 m/z, normalized collision energy of 27%), detected in the Orbitrap (R = 15,000 at 200 m/z). The dynamic exclusion of previously acquired precursor ions was enabled at 18 s.

Data Processing

Raw MS files was managed by MaxQuant software (version 1.6.0.16), MS/MS-based peptide identification was carried out with the Andromeda search engine in MaxQuant, Andromeda uses a target-decoy approach to identify peptides and proteins at an FDR <1%. As a forward database, rat protein database from NCBI was used. A reverse database for the decoy search was generated automatically in MaxQuant. Enzyme specificity was set to "Trypsin," and a minimum number of seven amino acids

were required for peptide identification. Default settings were used for variable and fixed modifications [variable modification, acetylation (Protein-N terminus) and oxidation (methionine), fixed modification, carbamidomethylation]. A label-free, intensity-based absolute quantification (iBAQ) approach was used to calculate protein quantification based on the area under the curve (AUC) of precursor ions. The fraction of total (FOT) was used to represent the normalized abundance of a protein across experiments. The FOT was defined as a protein's iBAQ divided by the total iBAQ of all identified proteins in one experiment. The FOT was further multiplied by 105 to obtain iFOT for the ease of representation. Missing values were substituted with zeros.

Multinomial Logistic Regression Model

Multinomial logistic regression is a multiclass linear classification method commonly used to classify multiclass categorical variables (22). In this study, drug treatments were regarded as dependent variables. The multinomial logistic regression model was implemented, using the Python Sklearn library v. 0.19.1 (23), to predict the sample categories. Initially, for each single-omics screening process, the data were stratified into training (80%) and testing (20%) data sets. A stratified sampling strategy, based on treatment, was used to determine the training–testing split, to obtain homogeneous subgroups. A mixed-effects modeling approach, using the omics data as fixed effects, was used; the characteristics of the rats, and of their organs, were used as random effects, because random effects accounted for variations between the rats and organs that might affect the response. "L2" regularization was used to prevent over-fitting: regularization strength was tuned through five-fold cross-validation. To evaluate feature selection robustness, the modeling process was repeated five times. In each iteration, the top features (500 for RNA-seq and 100 for protein data), ranked in order of their absolute coefficients in predicting each of the drug combinations (six combinations for RNA-seq and seven for protein data), were summarized into non-redundant sets (comprising 3,568 elements for RNA-seq and 1,157 elements for protein data). The resulting feature sets are thought to be highly associated with the drug treatment, and less related to the variables that are not the main focus; the selected feature sets were therefore used in downstream analysis. Pearson correlation analysis, and complete linkage hierarchical clustering analysis, were implemented to evaluate the stability of the feature coefficients, based on their similarity among the five iterations.

Weighted Gene Co-expression Network Analysis

A signed weighted gene co-expression network was constructed in each single-omics data set using the WGCNA R package (24). The adjacency matrix was first constructed by weighting the Pearson correlation coefficient of molecule pairs with an estimated power (β; 20 for RNA-seq and 16 for protein data), which preserves continuous intrinsic connections without defining hard thresholds. Next, the adjacency matrix was transformed into a topological overlap matrix (TOM), providing the proximity measure of network interconnectedness. This matrix (1-TOM) was used as the input for downstream average linkage hierarchical clustering. Co-expression models were defined as the branches cut dynamically from the hierarchical tree. The co-expression pattern of a module was represented by its eigengene, also known as the first principal component. The intramodular hub nodes in a given module were selected based on their correlation with its eigengene. Sub-networks consisting of hub nodes and edges with strong connections were visualized using Cytoscape (25); hub nodes were ranked based on their connectivity within the sub-networks.

Functional Enrichment Analysis

The ClusterProfiler package was used to implement functional enrichment analysis and visualization, based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (26). The biological functions related most significantly to high-altitude adaptation in the enrichment analysis were selected for visualization.

Statistical Analysis

Data are presented as mean ± standard error. Between-group differences were analyzed using Student's *t*-tests or one-way ANOVA, followed by a Fisher's Least Significant Difference test. $P < 0.05$ was considered statistically significant.

RESULTS

Validation of the HAPH Model

In the group exposed to hypobaric hypoxic conditions for 21 days, body weight decreased significantly from 463.83 to 355.57 g (**Figure 1A**, $P < 0.0001$). With prolonged hypoxia, mPAP increased gradually, until it was ∼3 times higher in the hypoxic rats (**Figure 1B**, $P < 0.0001$). RVHI was significantly higher in rats kept under hypoxia (**Figure 1C**, $P < 0.0001$). These results indicate that the model of HAPH was successful.

Responses of HAPH to Treatments

mPAP was significantly higher in the model rats. However, it decreased following selexipag, macitentan, and reoxygenation treatment (**Figure 1D**) and declined non-significantly following riociguat treatment. Because HAPH causes increased pulmonary artery pressure, it leads directly to RV hypertrophy. Therefore, RVHI increased significantly in group M, whereas it decreased in the selexipag, macitentan, and reoxygenation groups. Riociguat did not decrease this indicator (**Figure 1E**).

Organ- and Treatment-Specific Co-expression Modules Identified *via* Mixed-Effects Modeling

To avoid statistical noise, we use a mixed-effects model that uses RNA-seq data to model treatment effects. We use a model in which some of the genes can be used to accurately distinguish a sample from each treatment. As a result, we selected 3,568 genes and 1,157 proteins. In each model, the weights of these genes provide drug prediction vectors, which were used for correlation analysis. The drug treatments were highly correlated, and their effects were almost identical. This modeling method

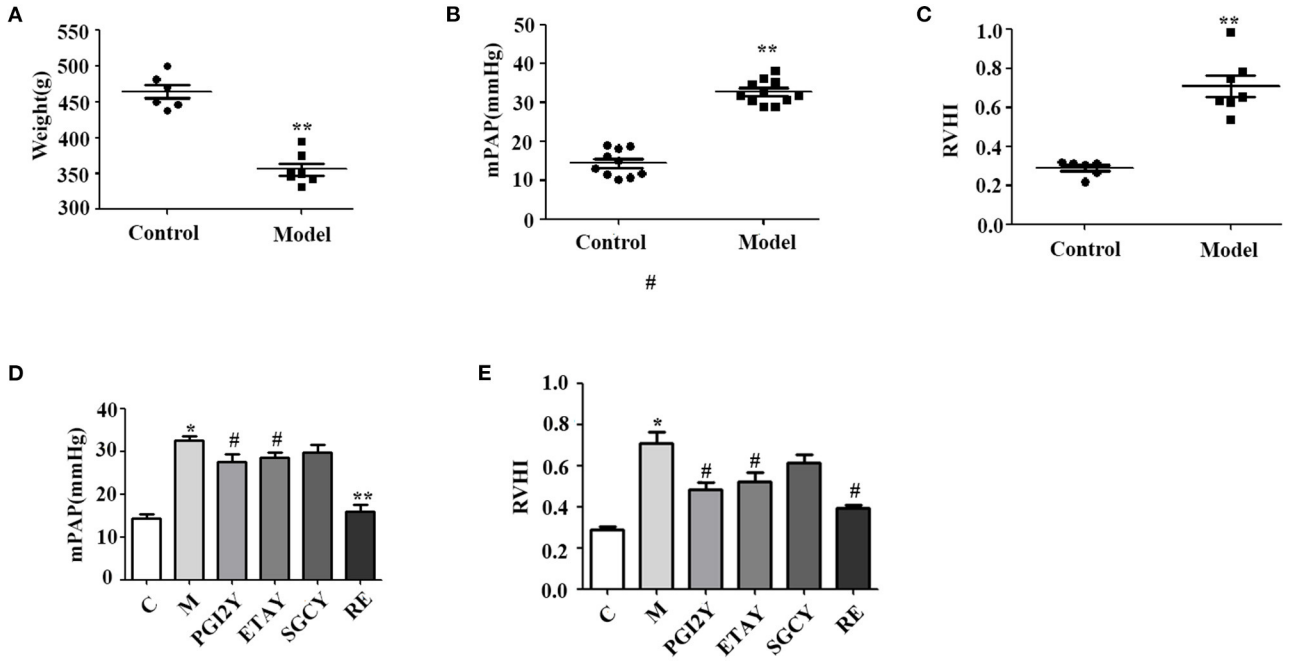

FIGURE 1 | Establishment of the high-altitude pulmonary hypertension (HAPH) rat model, and the effects of the targeted drugs and reoxygenation on high-altitude pulmonary hypertension. **(A)** Weight. **(B)** Mean pulmonary artery pressure (mPAP). **(C)** Right ventricular hypertrophy index: RVHI = RV/(LV + IVS). **(D)** mPAP and **(E)** RVHI after treatment. C, control; M, model group; PGI2Y, selexipag treatment group; ETAY, macitentan treatment group; SGCY, riociguat treatment group; RE, reoxygenation group. Data represent mean ± SD. Student's *t*-tests: $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. M; $^{**}P < 0.0001$ vs. M.

is stable, highly repeatable, and reliably identifies genes. The correlation heatmap and hierarchical clustering tree of the feature coefficients from the drug-treatment classification models (with random sub-sampling), for the RNA-seq data (**Figure 2A**) and protein data (**Figure 2B**), are shown; the color reflects the Pearson correlation coefficient.

We used WGCNA to obtain a co-expression network of known tissue-specific functional modules and selected the three co-expression modules most related to the organ sample types (**Figures 2C,D**): the different organs clearly have different molecular modules, and the LV and RV show consistent trends. For the RNA-Seq data, the black and green modules were most significantly correlated with the lungs. The magenta module was correlated with the heart. For the proteomics data, the turquoise module had the most significant correlation with the heart; the green module was correlated with the heart and lungs. The yellow module was correlated only to the lungs. The gene significance in these modules was imported into Cytoscape software to construct at the WGCN. MCODE was applied to filter the network module and select hub genes.

RNA-seq Analysis Identified the Key Pathways Altered in HAPH and the Treatment Conditions

Genes act in a coordinated manner to carry out their biological functions. Pathway analysis helps to better understand the biological function of genes. In order to confirm the potential pathway in high-altitude pulmonary hypertension and administration of targeted drugs, the KEGG analyses were performed. Therefore, we identified the key pathways altered in HAPH and affected by the treatments. GO and KEGG functional enrichment analysis for each module, based on RNA-seq data, revealed many molecular pathways that may be associated with HAPH and its related adaptive mechanism. GO-enrichment analysis revealed that, among the enriched differentially expressed genes were those involved in metabolic regulation, transcription, and translation. The pathways were mainly enriched in ATP, gtpase activity, transcription factor, unfolded protein reaction, Wnt, Notch, Apelin, hemoglobin, oxygenase active, the rRNA processing metabolism, while the mitochondria promotes apoptosis in the black and green modules (**Figures 3A,B**). The enriched pathways are those involved in metabolic process such as retinol metabolism, tyrosine metabolism and butanoate metabolism; regulation of blood pressure; myoblast proliferation and differentiation; adrenergic signaling in cardiomyocytes; mitochondria and Wnt in the magenta module (**Figure 3C**). The detailed informations of the biological processes (GO_BP), cell components (GO_CC), molecular functions (GO_MF), and pathways enriched in kegg (KEGG) enriched in the GO database by different modules are shown in the **Supplementary Table 1** based on the **Figures 3A–C**. We selected the core molecule in each module, to form the core gene sub-networks (**Figures 3D–F**; the color indicates the original module): the gene at the bottom of the figure is the most important, and importance decreases in

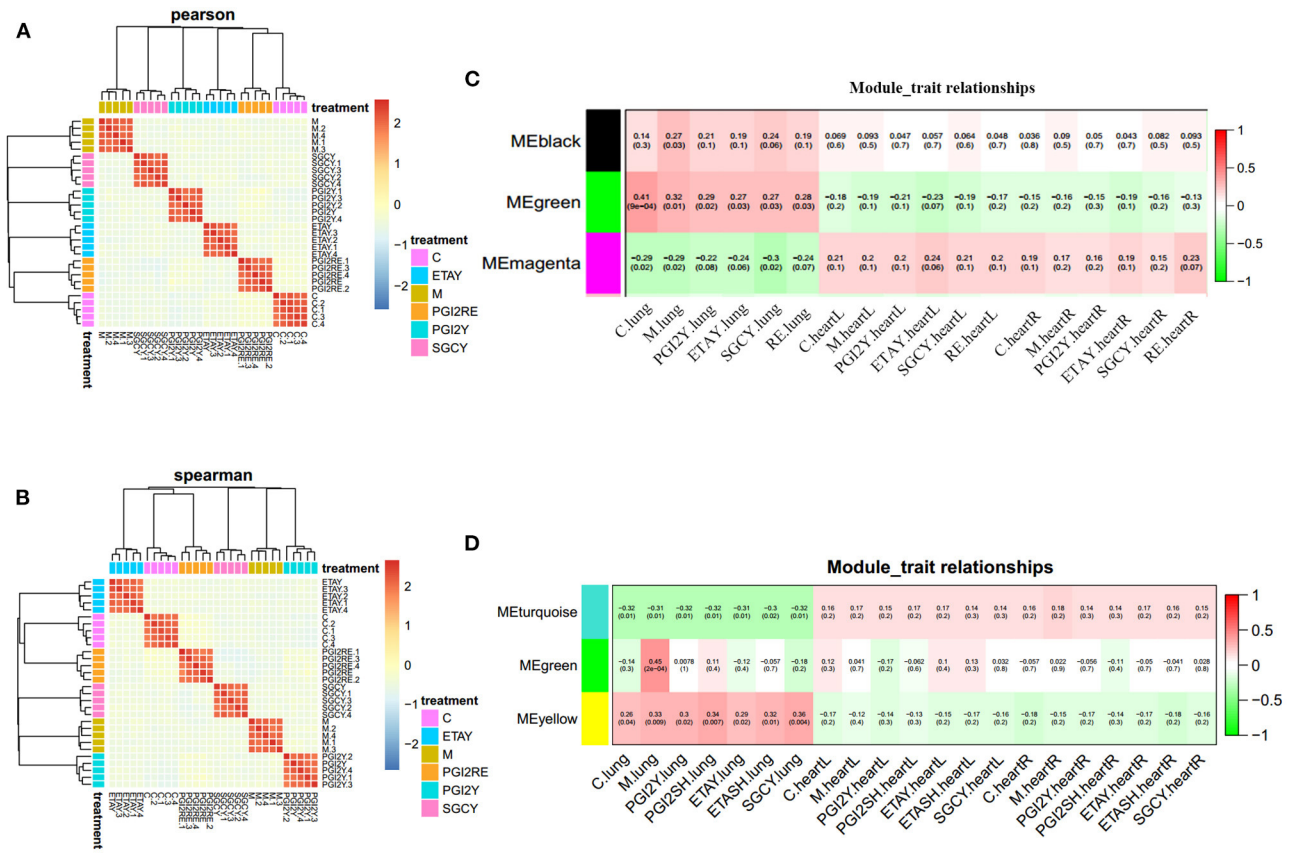

FIGURE 2 | Feature selection robustness validation, and identification of co-expression modules. Correlation heatmap and hierarchical clustering tree of the feature coefficients in the drug-treatment classification models (with random sub-sampling) of **(A)** RNA-seq data and **(B)** protein data. The color reflects the Pearson correlation coefficient. Organ-specific co-expression modules identified in **(C)** RNA-seq data and **(D)** protein data, using Weighted Gene Co-expression Network Analysis, and their correlation to sample category. The Pearson correlation is listed above its associated *P*-value in each square. The color of each square corresponds to the correlation (red, positive; green, negative; white, no correlation). The black and green modules in **(C)** represent up regulation in the lungs. The magenta module in **(C)** represents upregulation in heart. The turquoise module in **(D)** represents unique in heart. The yellow module in **(D)** stands for unique in lung. The green module in **(D)** represents sharing in the heart and lung.

a counterclockwise direction. The five most important genes (by module) were Bcr, Aox1, LOC100365697, Tshz1, and Rapgef1 (black module); Leng8, St3gal4, Slc51a, Efhc2, and Socs3 (green module); and Zfp346, Dnmbp, TP53bp1, Cacng7, and Ddn (magenta module). After selecting the hub genes, we further analyzed their effects by tissue, treatment, and organizational modules (**Supplementary Figure 1**): the radar charts each represent the effects of a molecule in an organization.

We also concluded that different modules may use some key pathways to extent. In the HIF-1 signaling pathway (**Supplementary Figure 2**), we found that the Cdkn1a, Vhl, Timp1, and Angpt2 genes were included in the black module. Eno4, Pfkfb3, Hmox1, Slc2a1, Rbx1, Rela, and Camk2g were shown in green module, while Hk1 was in magenta module. Referring to the TGF-β pathway (**Supplementary Figure 3**). LOC103691556, Bmp2, and Cul1 were included in the black module. Smad3, Acvr1b, Bmpr1b, Rbx1, and Thbs1 were chosen in green module. In magenta module, genes were Pitx2, Smad9, and Zfyve9. Regardless of the familiar pathways above, we also screened the Wnt signaling pathway (**Supplementary Figure 4**), Plcb1, Dkk2, Fzd3, Wnt5b, Dvl1, Wnt9a, Porcn, Fzd6, and Cul1were highlighted in black module. Apc, Rbx1, Notum, Prkacb, Vangl2, Smad3, Tcf7l2, and Camk2g were picked by the green module. However, the genes were Sfrp5, pc2, Ccnd2, Wnt9b, Dvl3, Nlk, Prickle3, Sfrp1, Cacybp, Sfrp2, and Cxxc4.

Differential Protein Expression Was Observed in HAPH and Under the Treatments

To identify the related signaling pathways and biological processes, we used GO analysis of the "biological process" category, and applied canonical pathway analysis to the turquoise, green, and yellow modules. The results show that pathways were mainly enriched in the processes of catabolism such as purine metabolism, D-Glutamine, and D-glutamate metabolism, fatty acid metabolism, oxoacid metabolic process and fructose and mannose metabolism;

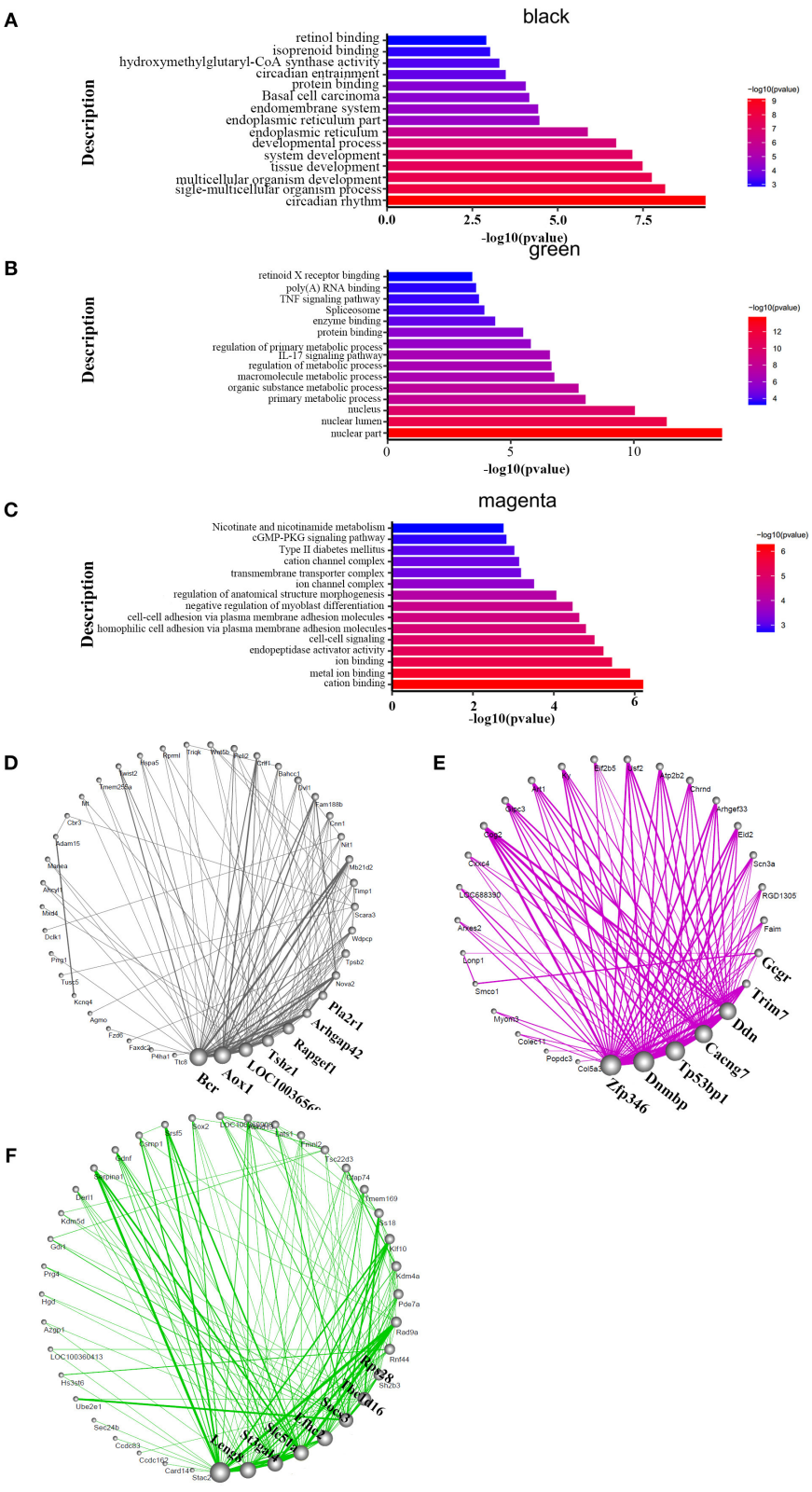

FIGURE 3 | Functional enrichment analysis and hub molecular identification of co-expression networks based on RNA-seq data. **(A–C)** Biological functions enriched in organ-specific modules. Bar length indicates the $-\log_{10}$ of the *P*-value from the Fisher's exact test. Edge width represents connectivity strength. **(D–F)** Organ-specific co-expression hub networks. Edge width represents connectivity strength. Node size represents the level of hubness.

catalytic activity such as organic substance catabolic process and cellular catabolic process; protein binding; carboxylic acid metabolism; fructose and mannose metabolism in the turquoise module (**Figure 4A**). The green module was mostly enriched in the processes of extracellular matrix components, nucleosome localization, histone H3-K4 trimethylation, histone H3-K27 trimethylation, and glutathione synthase activity in the green module (**Figure 4B**). Processes related to vesicles, exosomes, cell assembly, proteolysis, proteasome, and RNA transport were enriched only in the yellow module (**Figure 4C**). The detailed informations of the biological processes (GO_BP), cell components (GO_CC), molecular functions (GO_MF), and pathways enriched in kegg (KEGG) enriched in the GO database by different modules are shown in the **Supplementary Table 2** based on the **Figures 4A–C**. The organ-specific co-expression hub networks are shown in **Figures 4D–F**, and the relative expression levels of the organ-specific hub genes in the drug-treated corresponding organs are shown in **Supplementary Figure 5**.

Integrated Multi-Omics Analysis to Verify Key Genes

The differential gene and protein expression data was used to integrate and correlate the multiple omics analyses. We compared the core genes of the modules that exhibited the same trends in RNA and protein expression, and comprehensively considered the importance of the RNA and corresponding proteins and their related genes in their respective omics modules. We then selected the following genes as the most important genes from the two sets of corresponding modules: Serpina1, Ccar2, Rps28, Pxn, Enoph1, Sec24b, S100a8, Glod4, Emd, U2af2, Ephx2, Cryz, Pter, Chordc1, Nt5c2, LOC102550385, Acy1, Cops2, Cmc1, and Usp9x. These represent RNA and proteins that are highly consistently associated with the RNA and protein datasets, which are important in both the genomics and proteomics analyses, and that may play roles in gene transcription and translation.

The drugs differed in efficacy at the level of the important genes. Some of the drugs differed in these core molecules, and their therapeutic effects may differ. The hub molecules with consistent co-expression patterns in the genomic and proteomic datasets are labeled in **Figure 5**.

DISCUSSION

In our rat HAPH model, selexipag, macitentan, and reoxygenation significantly reduced mPAP, and riociguat reduced it insignificantly. Reoxygenation most effectively reduced mPAP, almost to the levels observed under normoxia. Similar results were observed for RVHI. As for the guidelines for the diagnosis and treatment, patients with PH who are hypoxaemic should receive long-term O_2 therapy (27). Similarly, Sime et al. (5) reported the the effectiveness of oxygen therapy for HAPH, hence we set the reoxygenation-treated group as the positive group. Considering the few researches available on the targeted drugs on HAPH, we applied the drugs for further exploration.

Bellaye et al. (28) showed that AdTGF-β1-induced pulmonary fibrosis in rats is accompanied by PH, however, macitentan mitigated the development of PH induced by reduced mPAP. Macitentan could also improve monocrotaline-induced pulmonary arterial hypertension hemodynamically and histopathologically (29). In addition, the effect of macitentan is more effective than bosentan (30). In European treatment guidelines, selexipag were recommended to use in patients with PH and WHO functional class (FC) II or III (31). In Honda's research, they induced a pulmonary arterial hypertension model in SD rats by injecting the vascular endothelial growth factor receptor antagonist Sugen 5416. They also exposed Fischer rats to hypoxic conditions to induce PH. Experimental results show that selexipag could greatly ameliorate the right ventricular systolic pressure and right ventricular hypertrophy in SD rats. In the article, the authors demonstrated how selexipag attenuated the proportion of lung vessels with occlusive lesions and the medial wall thickness of lung arteries. It also reduced RV hypertrophy and mortality caused by RV failure in the model Fischer rats (32). Interestingly, in this study all of the rats of the hypoxia model were returned to normoxia after hypoxia for 3 weeks including the rats taken selexipag. However, the rats in our study were under hypoxic conditions and selexipag treatment simultaneously. As a soluble guanylate cyclase stimulator, the effect of riociguat does not depend on the levels of NO in the body. It can increase the levels of cGMP in plasma alone or synergistically with NO, causing vasodilation and anti-remodeling effects. In a 10 years follow-up study, riociguat could improve pulmonary vascular resistance and cardiac index for up to 8 years, but failed to improve pulmonary arterial pressure (33). In a clinical trial of in patients with pulmonary hypertension caused by systolic left ventricular dysfunction, riociguat did not decrease the mean pulmonary artery pressure, yet it improved cardiac index as well as pulmonary and systemic vascular resistance (34). In our study, according to the effect of riociguat on HAPH, the recovery of mean pulmonary artery pressure was not observed. However, in another study of rats with induced PH by the vascular endothelial growth factor receptor antagonist SU5416 and hypoxia, the effect of riociguat was more effective than that of sildenafil on PH (35).

GO and KEGG functional enrichment analysis of the RNA-Seq and proteomics data sets revealed the organizational modules enriched in various pathways. There was little overlap in the differences in enriched categories identified using the RNA-Seq and proteomics data. However, three genes including Serpina1, Cryz, and CMC1 were consistently identified by the two data sets and should be further studied.

Using RNA-seq, we observed that the differentially expressed genes were involved in metabolic regulation, transcription, and translation. Proteomics studies the protein composition of cells, tissues, or organisms, and their responses to stimuli. Differential protein expression in tissue or blood samples reveals how proteins change during pathogenesis; identifying the corresponding genes and metabolites makes it possible to

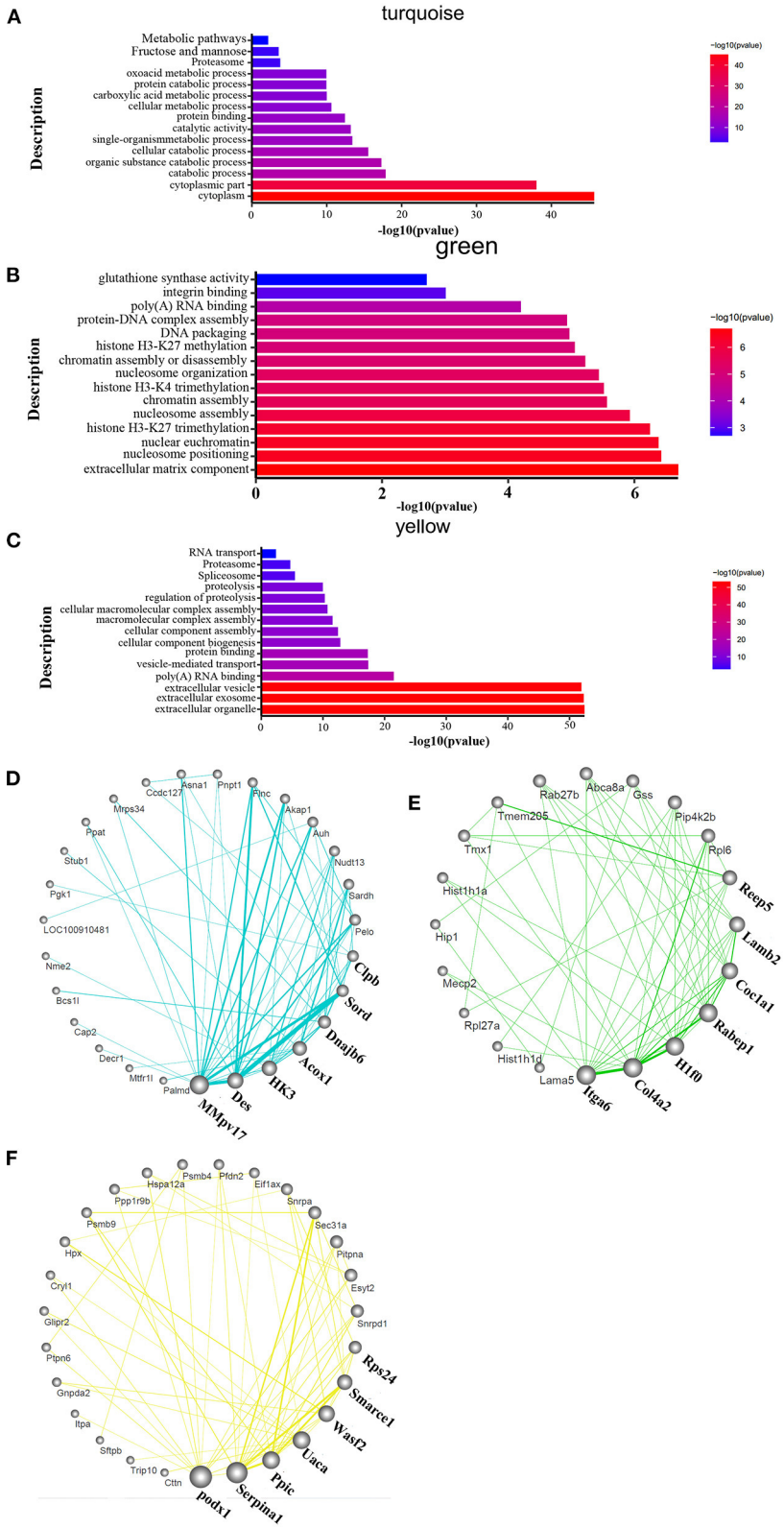

FIGURE 4 | Functional enrichment analysis and hub molecular identification of co-expression networks based on protein data. **(A–C)** Biological functions enriched in organ-specific modules; bar length indicates the $-\log_{10}$ of the *P*-value from the Fisher's exact test. **(D–F)** Organ-specific co-expression hub networks. Edge width represents connectivity strength. Node size represents the level of hubness.

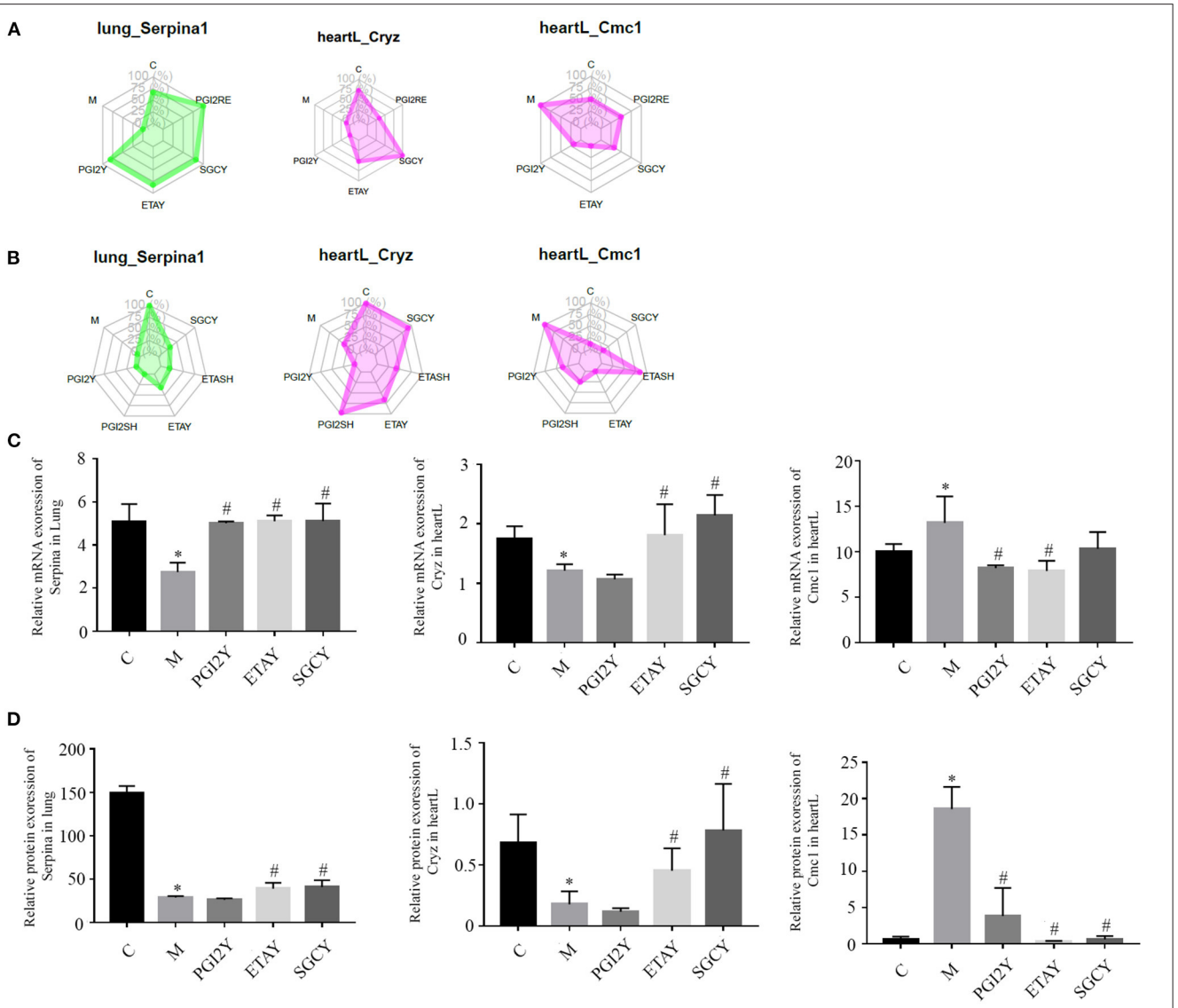

FIGURE 5 | Hub molecules with consistent co-expression patterns in the multi-omics datasets. Relative expression levels of Serpina1, Cryz, and Cmc1 in corresponding modules based on **(A)** RNA-seq data and **(B)** protein data. Relative expression of **(C)** mRNA and **(D)** protein, of Serpina1, Cryz, and Cmc1. heartL, the left heart. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. M.

study disease pathogenesis, diagnosis, and treatment. Using proteomics, the turquoise module was enriched mainly in the processes of catabolism, catalytic activity, protein binding, carboxylic acid metabolism, and fructose and mannose metabolism. The RNA-Seq and proteomics data produced different key GO terms related to differential expression. Absolute transcript abundance is often poorly correlated with protein expression levels; for instance, cardiac disease gene expression profiles had a limited commonality at the transcriptome and proteome levels (36). Similarly, our integrated approach, combining transcript abundance and protein turnover in HAPH, supports the notion that both transcriptional and post-transcriptional mechanisms affect pathogenesis in complex diseases.

Based on GO and KEGG functional enrichment analysis, the organizational modules were enriched in different pathways. Gtpase activity, in the black module, is an example of this. Rho, a small monomeric G protein, has gptase activity, and belongs to the Ras superfamily of proteins that regulate cell growth, differentiation, and survival (37). Rho participates in PH pathogenesis by promoting pulmonary vasoconstriction and structural remodeling. Rho-kinase inhibitors can induce acute pulmonary vasodilation, prevent PH, and induce pulmonary vascular remodeling (38–40). During PH pathogenesis, transcription factors are involved in the mechanisms of PH and of targeted treatment.

The unfolded protein response (UPR) process is mediated by three transmembrane receptor proteins in the endoplasmic

reticulum (41). Low-pressure and low-oxygen conditions induce this process (42). Both endoplasmic reticulum stress and the UPR may play important roles in PH pathogenesis (43, 44).

HIF-1 is a heterodimer of HIF-1a and HIF-1b. It is a key regulator of oxygen homeostasis, and it could accommodate the adaptive molecular response under hypoxic conditions (45). The mechanism of HIF-1 in hypoxia-induced pulmonary hypertension has been clarified. It participated in the pathophysiologic alterations of both smooth muscle and endothelial cell biology in patients with PH (46). Further, it promotes vascular post-injury remodeling in both pulmonary and systemic arteries. Finally, it resulted in the apoptosis of pulmonary arterial smooth muscle cells and alleviation of pulmonary vascular remodeling when suppressing HIF-1 (47).

In our RNA-Seq analysis, the Notch pathway was screened. Notch proteins are cell membrane receptors that mediate signaling between cells, and hence play an important role in cell-to-cell communication (48). In the vascular system, the Notch pathway is involved in vascular development, angiogenesis, and arteriovenous specification. All of the screened pathways were related to HAPH. These pathways provide new targets for the treatment of HAPH.

After screening, we identified the three most important genes, Serpina1, Cryz, and CMC1, using the multi-omics datasets. Serpina1 encodes a serine protease inhibitor whose targets include plasmin, elastase, thrombin, trypsin, chymotrypsin, and plasminogen activator. This protein is secreted in the liver, the bone marrow, by lymphocytic and monocytic cells in lymphoid tissue, and by the Paneth cells of the gut. Serpina1 is a major circulating antiprotease. Pathogenic mutations in SERPINA1 gene will lead to α1-antitrypsin deficiency (AATD). α1-antitrypsin (AAT) was first discovered in 1963, and it was related to hereditary emphysema (49). Under normal homeostatic conditions, AAT could prevent damage of the lung alveolar matrix by regulating the proteolysis of human leukocyte elastase. AAT is secreted into the blood plasma, but its primary site of action is the lung parenchyma, despite it was secreted into the plasma (50). Many studies have proved that the up-regulated expression of AAT in monocytes will prevent the protease destruction in the lung microenvironment. This process will be regulated by the bacterial endotoxin and/or early production of inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor a (TNF-a) in the lung. Chronic obstructive pulmonary disease (COPD), emphysema, PH, pulmonary fibrosis, and chronic liver disease are related to Serpina1 deficiency (49). Furthermore, Hou et al. (51) reported that the interacting effect between lncRNAs and mRNAs on the pathogenesis of PH, in which the mRNA of Serpina1 was included. In our study, we found that Serpina1 played an important role in HAPH, hence it is worthy of further study.

Cryz encodes the crystallin zeta. The z-crystallin was found at first in the lenses of guinea pig (52). Crystallins are separated into two classes: taxon-specific, or enzyme, and ubiquitous. Cryz encodes a taxon-specific crystallin with NADPH-dependent quinone reductase activity distinct from other known quinone reductases. Cryz protein has a potentially pivotal role in cancer, allowing cells to hijack or subjugate the acidity response mechanism, to increase their ability to resist oxidative stress and apoptosis, while fueling their glutamine-addicted metabolism. However, CryZ protein was firstly discovered for its ability to bind DNA in cellfree settings (53). Later, Curthoys' group provided strong evidence that CryZ is an mRNA-binding protein. In a renal cell model, CryZ stabilizes rat glutaminase (GLS) mRNA (54). This protein binds specifically to adenine-uracil-rich elements in 3′-UTR of mRNA, for example bcl-2 and it has been reported to act as trans-acting factors in the regulation of certain mRNAs (55, 56) When it binds to bcl-2 mRNA, it will enhance the stability and effect of bcl-2 mRNA. In the Qi report, the researchers conducted a genome-wide association (GWA) study on circulating resistin levels in European individuals. The results indicated that novel loci near the TYW3/CRYZ gene (1p31) was associated with resistin levels. The resistin-rising allele (C-allele) of TYW3/CRYZ SNP rs3931020 was associated with increased coronary heart disease risk (55).

CMC1 encodes C-X9-C motif containing 1, which interacts and instantly stabilizes the early COX1–COX14–COA3 complex. CMC1 is regarded by some to be a COX1 chaperone. In a CMC1-knockout cell line, COX1 was able to synthesize normally, whereas mitochondrial respiratory chain complex IV (CIV) activity decreased (57), due to the instability of the newly synthetized COX1. As it is known, in the chronic thromboembolic pulmonary hypertension model, the impaired mitochondrial respiratory function participated in the development of right ventricular dysfunction. In addition, in the mitochondria containing 30–40% of the heart, CMC1 plays a vital role in the rat HAPH model. The drugs had different effects on these three core molecules; some of these effects may indicate therapeutic benefits. The three genes were the first engaged in the mechanism of pulmonary hypertension.

RNA-Seq and proteomics methods can effectively reveal genes or proteins related to hypobaric hypoxia-induced pulmonary hypertension. Using the GO function and KEGG pathway enrichment analysis of differentially expressed genes, we identified multiple pathways related to HAPH, and that responded to the targeted drugs. HAPH is a complex disease. Our findings show that reoxygenation and drug therapy can rescue abnormal gene expression, and restore affected pathways, in rats under simulated high-altitude hypoxia, thereby playing a role in myocardial protection.

CONCLUSION

Selexipag, macitentan, and reoxygenation significantly attenuated the rat HAPH model, and riociguat had a weaker effect. Differentially expressed genes were involved in metabolic regulation, transcription, and translation. Certain proteins were affected by HAPH and by the treatments. We screened the key genes, Serpina1, Cryz, and Cmc1, using a multi-omics approach. These findings may provide a better understanding of the molecular mechanisms involved in hypoxia and may provide new therapeutic targets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of the Chinese PLA General Hospital.

AUTHOR CONTRIBUTIONS

CL and KH took responsibility for the integrity of the data, the accuracy of the data analysis, and designed the concept and obtained funding. XX, HL, and QW drafted of the manuscript, executed the experiments, did statistical analysis. XL, YS, GG, and YC carried out the data collection. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Key Technologies R&D Program for New Drugs of China (Grant Number 2018ZX09J18109-004) and National Key R&D Program for Precision Medicine (Grant Number 2017YFC0908400).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.742436/full#supplementary-material

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frontiers in Medicine

ORIGINAL RESEARCH
published: 23 November 2021
doi: 10.3389/fmed.2021.728529

Perspectives, Expectations, and Concerns of European Patient Advocates on Advanced Therapy Medicinal Products

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This paper presents the results of a qualitative study based on semi-structured interviews of 10 expert patient advocates on several different issues around Advanced Therapy Medicinal Products (ATMPs). The interviews were conducted between February and May 2020 based on a guideline with a list of 8 topics that covered concerns about safety and ethics, access problems and limitations, pricing of ATMPs and educational needs for patient communities. Overall, the interviewees expressed a high degree of convergence of opinions on most of the topics and especially on the identification of the reasons for concern. Conversely, when asked about possible solutions, quite a wide range of solutions were proposed, although with many common points. However, it highlights that the debate is still in its infancy and that there are not yet consolidated positions across the whole community. A general concern emerging from all the interviews is the potential limitation of access to approved ATMPs, both due to the high prices and to the geographical concentration of treatment centers. However, patients recognize the value of a model with a limited number of specialized clinical centers administering these therapies. On the ethical side, patients do not show particular concern as long as ATMPs and the underlying technology is used to treat severe diseases. Finally, patients are asking for both more education on ATMPs as well as for a more continuous involvement of patient representatives in the whole "life-cycle" of a new ATMP, from the development phase to the authorization, from the definition of the reimbursement scheme to the collection of Real Word Data on safety and long-term efficacy of the treatment.

Keywords: gene therapy, cell therapy, patient advocacy, ethical analysis, tissue therapy, patient—centered care, bioethic

OPEN ACCESS

Edited by:
Nuno M. Neves,
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Reviewed by:
Antonio Vallano,
Catalan Health Institute (ICS), Spain
Joaquim Vives,
Banc de Sang i Teixits, Spain

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *21 June 2021*
Accepted: *01 November 2021*
Published: *23 November 2021*

Citation:
Benvenuti S, Wang CM and Borroni S (2021) Perspectives, Expectations, and Concerns of European Patient Advocates on Advanced Therapy Medicinal Products. Front. Med. 8:728529. doi: 10.3389/fmed.2021.728529

INTRODUCTION

New scientific progress in cellular and molecular biotechnology has led to a new field of biomedicine which offers new opportunities for the treatment of diseases and dysfunctions of the human body (1–6). Advanced therapy medicinal products (ATMPs) are the treatments resulting from the advancement in this new field and they ≪may be used in or administered to human beings with a view to restoring, correcting, or modifying physiological functions by exerting principally a pharmacological, immunological or metabolic action≫ (7, 8). According to the European Regulation (EC) No 1394/2007 (7) and the Directive 2001/83/EC (8) ATMPs are medicines for

human use that are based on genes, tissues or cells; a similar definition, although not identical, is adopted by the United States Food and Drug Administration (FDA) (9).

Despite the large number of ATMPs in development (10), currently, only 20 ATMPs have been approved by the FDA (11) and even fewer by the European Medicines Agency (EMA). The products under development are intended to treat a wide variety of conditions, spanning cancers, inherited diseases, and other chronic conditions (10).

A number of technical challenges are still open, both in the development of such products (12–17) as well as in the pricing and access to these therapies, including ethical and economic issues, follow up management, logistic and delivery issues, and equity of access (18–22). Expert patient advocates could provide important insights into these discussions, especially on ethical and access issues (23–31). Patient perspectives are crucial, especially when considering the need to define the real value of these therapies in terms of improvement for quality of life (QoL) and thus, a fair price (30, 31). Patient contribution in the collection of follow-up data is also crucial to provide information on the effectiveness of the therapies on a long-term and real-world basis (32, 33).

In addition, specific inputs from patients are needed on organization of healthcare system and clinical centers for the delivery of ATMPs as well as organization of clinical trials operation (26, 28). These inputs are specifically useful to contribute to the definition of the best ATMP delivery model to manage cross-border mobility issues and facilitate patients' access to therapies.

For these reasons, it is paramount that academia, industries, health systems, regulatory, Health Technology Assessment (HTA) bodies and payers involve patients in the processes of research, development, approval, pricing and marketing of ATMPs (28).

For the adoption of ATMPs as a part of treatment plans, patient empowerment will be essential. Educational activities on specific topics will be fundamental to enable patients to approach ATMPs with realistic expectations of the risks, potential benefits and to participate in clinical trials with increased awareness and the necessary basic knowledge to take informed decisions (30, 34, 35).

In this paper, we have gathered perspectives from patient advocates on a number of different issues, such as relevance for patients of a discussion on ATMPs, barriers and solutions to improve access to specialized centers, pricing, feasibility, and relevance of the collection of long-term follow-up data. Also included were particularly sensitive issues such as equity of access, safety and ethical concerns and the value a patient recognizes for a treatment that might change the course of the disease, including issues related to the sustainability of national health systems.

METHODS

Interviewees

Interviewees were selected based on their proven track of patient advocacy, reputation, geographical area, and disease area representations. In addition, they should represent umbrella patient organizations, single patient organizations, independent experts, or is a patient themselves. Patient advocates who had undergone training on medicine research and development or patient academies were preferred. Patient advocates are appropriate interviewees because they are informed and are visionary trend setters for their disease areas. This makes the idea for interview with respect to the general patient population. Ten patient advocates were selected and invited for interview for their opinions and qualitative study. The interviewees located in different geographical areas within Europe (Belgium, Germany, Ireland, Italy, Netherlands, Poland, Spain, Sweden) and several disease areas (cancer, rare disease, multiple sclerosis, diabetes, Parkinson's disease, hemophilia). Whether an ATMP was available or not for their diseases of interests was not taken into consideration for the selection. With the explicit agreement of all interviewees, a full list of their names and affiliation is included in the Acknowledgments. No compensation was foreseen for the interviewees.

Interview Guide and Methodology

The methodology for the interviews was developed based on standards for qualitative research (36, 37). The interview guide was developed within the project RESTORE, a European Commission funded project (Grant Agreement number: 820292). A guideline for the interview with a list of 8 topics was developed and piloted in 2 mock interviews with Italian patient advocates. The responses of the mock interviews are not included in the results. The interview covered the following topics: (1) Relevance for the patients of a discussion about ATMPs; (2) Barriers and solutions to improve patient access to specialized centers qualified to administer ATMPs; (3) Pricing; (4) Feasibility and relevance of the collection of long-term follow-up data after treatment; (5) Access pathways; (6) Safety concerns; (7) Ethical concerns; (8) Education and training needs for patients. This interview guide was sent to the interviewee together with the consent information sheet before every interview (**Supplementary Materials**). The guidelines were shared beforehand to facilitate the conversation during the interview and reduce any possible anxiety in interviewees who may otherwise have felt under examination.

Data Collection

The interviews were conducted between February and May 2020. Each interview lasted 30–45 min and was recorded. Before starting the interview and the recording, the interviewer presented the main points of the consent sheet. Specific emphasis was put on the fact that the interviewee can skip any question/topic and stop the interview at any time. Most of the interviews were run by an expert patient advocate (co-author of this report) to make interviewees feel comfortable being in a conversation with a peer.

The transcript of the recording was sent to the interviewee for cross-check, validation and to avoid unintended bias by the interviewers. The recordings are kept as confidential and any opinion expressed during the interviews is reported under the so-called Chatham House Rule (38). Quotations from specific

interviews are included in the results without references to the interviewee who expressed them to preserve the confidentiality of the opinions expressed.

RESULTS

The results are presented in 8 paragraphs following the structure of the interview guidelines:

Relevance for the Patients of a Discussion on ATMPs

Almost all those interviewed (9/10) agreed that ATMPs are a "hot topic" for patient communities.

Some of them underlined that this is a "hot topic" especially for:

- Specific disease areas, for instance blood disorders, cancers and diseases with genetic origin / rare diseases.
- Diseases where, currently, there are no treatments available.

For diseases where treatments are available, even if the disease is chronic and lifelong, if persons are able to have a good quality of life, there is less excitement about ATMPs than in diseases where this is not the case.

In addition, according to some of the interviewees, the interest in advanced therapies is due to the fact that they may target the root causes of the diseases. Thus, for some disease areas there is the expectation that a curative solution might be possible. The large number of currently ongoing clinical trials with cell and gene therapies has attracted the interest of patients. They are especially keen to learn more about these advanced therapies; how they work, how patients will benefit from them and when they will be accessible.

Almost half of the interviewees (4/10) agreed about the importance of offering patients good quality and targeted communication about ATMPs, especially on:

- The mechanisms of action of gene therapy and of the adopted viral vector.
- The benefits and risks, focusing also on the potential side effects (safety).
- A better understanding of which diseases could potentially be treated with ATMPs in order to avoid high and unrealistic patient expectations.

Barriers and Solutions to Improve Patient Access to Specialized Centers Qualified to Administer ATMPs

Eight out of ten interviewees think that the creation or recognition of specialized clinical centers is the best model for delivering ATMPs. The major reasons that lead to the preference for this model are the following:

- treating a greater number of patients will allow the centers to gain experience in administering ATMPs but also in the management of the possible toxicity of these therapies that might put patients at risk;
- in smaller centers with fewer patients treated and consequently less expertise, some side-effects may be missed or treated in such a way that it might negatively affects the final treatment outcome;
- in a model with several centers in every country, geographically distributed and treating a relatively low number of patients, some concerns may arise about inconsistency in treatment delivery, in monitoring treatment response over time and the difficulty of sharing data;
- the centralization of real-world data is crucial for the success of ATMPs, not just scientifically, but also clinically. Due to the initially small number of patients likely to be treated, it will be imperative that data is shared to obtain maximum benefit for the majority of patients. One of the main concerns raised regarding data sharing was indeed the risk that valuable data might be collected in isolation without sharing between other hospitals and principal investigators;
- the delivery of some of these therapies require special devices, specific clinical settings and the certification of hospitals to administer the treatment;
- having specialized centers is beneficial for patients themselves, for clinicians, regulatory authorities and pricing and reimbursement authorities who can rely on a form of quality certification that ensures uniformity and quality of the treatment.

Few interviewees (2/10) highlighted the fact that ATMPs could increase inequality in a patient population as the need to administer the treatment in a limited number of specialized centers will constitute an additional barrier to access. Not every patient can travel to get treated, therefore some patients in certain areas might not be able to reach the centers of excellence and thus will not have access to the treatment they need. From a citizens' perspective, this is not acceptable.

The common opinion is that these therapies (or at least some of them) need to be centralized in experts centers at EU level but there is also a need to create a support system that allows patients to access those centers no matter where they live.

Interestingly, two of the interviewees suggested pricing ATMPs as a service. According to this model, industries should consider providing a full service, including both the drug and the clinical costs for its administration as well as paying attention to include patients from different locations in the marketing plans. They also need to quantify how these products are increasing the QoL of the patients in a much broader way. In the end, everything should be revaluated considering ATMPs as a service not simply as a drug.

Pricing of ATMPs

The majority of interviewees (7/10) agreed that high prices of ATMPs are an issue and, in some cases, could be an obstacle for access to the therapy. The main concern is that high pricing leads to prolonged discussion and negotiation with Payers on reimbursement, slowing down access for patients. Some interviewees (5/10) also highlighted that in the long run, high prices could threaten the sustainability of national health care systems.

Almost half of the interviewees (4/10) report that there should be transparency on how prices are set, on the components to be assessed and on how the incentives are defined. The lack of clarity in the definition of prices could lead to inequalities between the prices of ATMPs and the prices of other lifesaving health treatments such as surgery. They recognize pricing is a complex process and believe both costs and value should be taken into consideration. The price may be linked to the costs of the therapies and to the incentives received by the developers, and even more importantly the need to focus on the value of the treatments. The definition of value should include not only the direct benefit of the treatment but also the cost saving due to the effect of that treatment on the progress of the disease and, consequently, on the burden of the disease.

Most agree that the discussion on the price of treatments should be done in a framework considering all the costs needed to deliver the treatment to patients, meaning costs of follow up and clinical data registry, screening, organizational costs to deliver the therapy to patients (professionals, specialized nurses, and special settings) and any other additional cost to get the real cost of the treatment for patients.

Interviewees expressed different opinions commenting on the innovative agreements recently signed between industries and payers to enable access to ATMPs. These innovative agreements include several different schemes designed to find a balance between the high cost of ATMPs and the uncertainty on their efficacy, including their long-lasting effectiveness; such schemes are generally referred to as Outcome Based Managed Entry Agreement, Risk Sharing Agreements, Value based price and Delay payment (20, 39–41). Managed entry agreements can facilitate patient access to treatments and are especially important for life-saving treatments where patients with high unmet needs should have early access. In many other cases, Managed Entry Agreements do not solve the issue of high prices in the long term. In addition, it may prove difficult for the competent authorities to apply them, especially for those new treatments where it could be difficult to evaluate the efficacy over a long-term period. For value-based price the definition of "value" may be controversial. The current limitation is that, so far, nobody can really say whether and for how long a treatment is going to work. Finally, delayed payments are not considered by the interviewees as a solution for high prices as the payment, sooner or later, will still impact heavily on healthcare budget.

According to the interviewees, the most promising instrument that has not yet been frequently used is pooling procurement among countries; a greater number of patients would give competent authorities a higher bargaining power when discussing the price.

When value-based price was discussed, interviewees were asked what "value" means for them. Most interviewees indicated that, for them, value in this context means the value for patients, which should be assessed based on data provided by patients: improvement in quality of life and safety. Patients themselves should be involved in providing data as well as in defining what should be intended with "value for patients." This means spelling-out the reasons why a certain product should be reimbursed and what benefit it could provide to the patient community. This value should be strictly linked with the improvement in the quality of life of patients. In addition, for most ATMPs, long-term follow-up assessing the safety of the product is recommended. For all the above-mentioned reasons, patients should work with Payers and HTA Agencies, with the final aim of contributing to the definition of standards in outcome measures and to the set-up of post-marketing registries.

Moving back to the development phase of the drug, value is defined as the measurable benefit during clinical trials. A strong statement by most of the interviewees is that, where possible, the endpoints of clinical trials should focus on the patient and not on the product. This means, for example, setting up a single platform for controlled clinical trials on a specific disease to evaluate and directly compare in the same trial different compounds from different companies. This will allow the direct evaluation of the value of each single treatment and the real benefit for patients.

Feasibility and Relevance of the Collection of Long-Term Follow-Up Data After Treatment With ATMPs

Interviewees suggest that patients tend to be generous in giving their data, also after the treatment, for the sake of research and for the benefit of other patients. However, half of the responders (4/8) indicated that it could indeed be difficult to involve patients in data collection for long-term follow up. All agreed that engagement of patients and their family is a critical factor for the success of long-term data collection.

Interestingly, some of the responders (3/8) suggested that a possible way to involve more patients in providing long term follow up data, could be to involve patient representatives in defining patient reported outcome measures (PROMs) and the questionnaires often associated with long-term follow-up and the assessment of patients' quality of life. In their opinion, patients will generally be more willing to answer questions that are meaningful to them.

Thus, it is important to develop together with patients a set of measures, asking fewer questions but that are more relevant to them and closer to their unmet needs in daily life. Having patient representatives involved in the design and definition of PROMs could also provide the scientific community and the regulators relevant insights on the most pressing unmet needs for the patient community.

Another point of view is that patients need to be motivated to share their data: patient engagement should be seen as a two-way exchange where patients provide value (data) and receive value in return. Therefore, it is necessary to determine how to give something back to patients in order to demonstrate that the investment of their time is worthwhile. On this point, interviewees suggested that patients should be provided with information both at a cohort level but also at individual level to be able to calculate how far they are from "the mean." Knowing to which percentile a patient belongs could empower them to either better accept the condition or to take action and look for further therapeutic strategies that could improve their quality of life. Another possibility would be to lower the cost of treatments for those who are engaged and compliant in data collection.

Finally, it is crucial to give feedback to patients about how the data are used. Some of the respondents suggested that to reduce possible concerns and encourage patients to share their data, good supervision and a good data management framework is necessary. This includes transparency about the use of data, who is going to use them and how patients can withdraw their consent in the event they no longer want to share their data. Information should be given about servers where the data are stored and their compliance with the European General Data Protection Regulation (GDPR).

Patient organizations could play a role in preventing patients dropping out of long-term post-treatment data collection by providing education about the importance of having the data to demonstrate the value of the treatments. It should also be explained to patients that lack of data about the value of the treatments could lead to later access to the therapies. Another point raised by one of the interviewees is the role that patient organizations could and should have in collecting data. This interviewee considers it very important that patient organizations act as the preferred channel to link patients and the competent authorities. This will ensure that patients reach competent authorities without any filter by individual clinicians or by the industry. Information is of value to citizens and in the current times, data are becoming a new currency.

Most of the respondents recognized that there are no longer technical barriers to the engagement of patients; data can be provided remotely and thus, it is no longer necessary to go back to clinical centers for every follow-up data collection point. Data collection can be done by remote monitoring, via mobile apps or organizing conference calls with patients at home. Upon direct questioning about data sharing, 6 out of 7 respondents agreed that patients are more likely to be willing to share their data than they are to be concerned about it. This is especially the case in extremely rare diseases, where patients hope that sharing their data could stimulate researchers to start studying their diseases and eventually improve their condition.

Concerning data collection, some additional interesting ideas are long-term and stable data collection not linked to a specific product but rather to a condition or a group of diseases, which could help in better assessment of the standard of care and its value as well as to establish a baseline for the assessment of old and new products. To facilitate that process, with the contributions of different pharma companies, national funds dedicated to set up and maintain the registries needed for pharmacovigilance should be created.

Access Pathways for ATMPs

The interviewees were asked about the appropriateness and duration of the processes for the approval of Clinical Trials, for Marketing Authorization and for price negotiation of ATMPs. Almost all the interviewees (8/10) thought that the process required for getting a treatment on the market takes too long.

Three interviewees suggested that the approval process should differentiate according to:

- The different nature of the therapies: autologous cells therapies versus "off the shelf" products. For example, autologous cell therapies are considered less risky for patients, thus the process could be faster.
- The different disease areas: high prevalence diseases vs. rare and complex diseases and diseases where there are treatments vs. diseases where there are not.

With regard to risk assessment in the approval process, it should be considered that this process has been developed for high prevalence diseases, therefore not considering that, in rare diseases, where the condition is severe and debilitating, the risk that patients are willing to take is higher.

While safety considerations are paramount for some of the interviewees (4/10), another suggested that the concept of acceptable risk, as well as safety, should be reconsidered taking into account the specific disease or patient situation. In accordance with this last comment, the risk assessment in the approval process should be reviewed because, in severe and life-threatening diseases, the level of risk that patients are willing to take is higher than in diseases where an alternative therapeutic option is available. For this reason, a different framework of approval for different diseases with different unmet needs should be created. As an example, one of the interviewees mentioned the "Right to try" model, signed into US law May 30, 2018. This could be helpful to patients who have been diagnosed with life-threatening diseases or conditions, who have tried all approved treatment options and who are unable to participate in a clinical trial. The combination of these conditions should allow them to access unapproved treatments that have completed Phase I.

With respect to the price negotiation process, the majority of the interviewees (6/10) agree that it should be improved and accelerated, while a few of them (3/10) commented about the fact that EMA is already taking action in order to accelerate approval processes—i.e., conditional approval, Prime medicine, etc. One of the possible actions suggested by the interviewees to accelerate the access to treatments, is to provide immediate access for certain patients and pre-file a price that can then be corrected after the negotiation. These are the key concepts of an early access scheme already used in France and called the Authorization of Temporary Usage (ATU) (42).

With respect to the speed of access to treatments, considering the limited number of patients included in the clinical trials, some of the interviewees mentioned the importance of having a robust system for the collection of long-term follow up data. This will allow for combining the need for patients to get access to a hopefully life-saving treatment and the need for additional data to evaluate whether that treatment is really providing a significatively higher therapeutic value. On one side the involvement of academia and a system of supranational cooperation have been mentioned as possible way to set up this system for real word data collections; on the other hand, it needs to set up *ad hoc* committees providing ongoing reviews of ATMPs already on the market to look at how efficacious the treatments are in the long term and in the real word setting. In the view of respondents, these committees should include patients, clinicians, regulatory agencies, HTA experts and payers.

Safety and Concerns on Unauthorized Treatments

Interviewees were consulted on patients' view on all the different unauthorized treatments available on the market. In this question, unauthorized treatments refers to treatments offered outside any legally authorized frame; therefore, for the purpose of this question, EMA authorized products as well as investigational products in an authorized clinical trials or administered under compassionate use were all considered "authorized" (43).

In general, all the interviewees agree about the fact that, especially for life threatening diseases that have no treatment options, patients are more willing to try any sort of treatment, overcoming fears about adverse events. In these cases, patients may be more likely try to obtain any available treatment, even if not authorized, and are willing to pay out of pocket for it.

One of the interviewees also reported that the long waiting time between EMA authorization of the product and its availability for patients due to long HTA and price negotiation procedures, might, in some cases, be one of the causes that push patients toward looking for unauthorized treatments.

Among the respondents, there are different opinions about how to counteract the diffusion of unauthorized treatment. More than half (5/8) think that patients should be better educated about ATMPs. More specifically, proper information is needed about what is on the market and what, if any, are the alternatives. In addition, patients need to know more about the long and complicated path of medicine development and how important it is to be treated in approved centers. According to one of the respondents, patient organizations should also work to empower the patients who may not quite understand the science, to weigh up the risk before going for any treatment they can find. Others (3/8) highlighted that local governments should take actions to counteract false information while, at European level, common rules are needed to sanction those who administer unauthorized treatments.

Ethical Concerns

Half of the interviewees agrees that there should not be any ethical barrier around therapies that alter the gene without transmitting this modification to the germinal line. A common opinion is that debate on ethical concerns is still at a very early stage. What is strongly affirmed by all interviewees is that all the relevant stakeholders, including patients, should be involved in all discussions about ethical limits. In this respect interviewees highlighted the importance of influencing the public debate on ethical issues to shift the focus toward health benefit for patients, as, according to them, this is the most relevant topic.

In addition, interviewees highlighted the need for more information about what new techniques such as gene editing can and cannot achieve, what the consequences could be and what are the hypotheses. There is a common feeling that it is not currently possible to define the real limits of these technologies, but it is important to determine how the application of these new techniques could change the course of certain diseases. Interviewees agree about limiting the treatments to the cure of genetic disorders avoiding any attempt to modify other physiological characteristics (e.g., eye color, height, etc.).

Finally, some respondents would like a common position to be elucidated on what happens if other less regulated countries develop and make gene editing techniques available to patients before they become available in Europe. Would this lead to European patients traveling abroad for curative treatments?

Education and Training Needs for Patients

All the interviewees agree on the need for educational tools for patients. Half of them (5/10) also mentioned the need to educate professionals, such as general practitioners, specialists and pediatricians. Only a few (2/10) mentioned the need to educate the general population and interestingly the need to educate policy makers (reimbursement agencies). According to two of the interviewees, the education actions should be addressed to people who are interested in learning about these therapies and should contain only topics that are directly relevant to that audience. In general, they underlined the need for more well-trained expert patients to be involved in development and marketing pathways of medicinal products. Consequently, there is the need for comprehensive training for expert patients in medicine development, approval, reimbursement and HTA. This is essential.

There are two major training focuses identified by the interviewees:

- Train patients specifically on ATMPs, explaining:
  - The differences between ATMPs and other medicinal products currently in use.
  - The differences among the different classes of ATMPs. This means for example explaining the difference between gene therapy with adeno virus and CAR-T cell therapies. Trainees need to understand the specificity of each class of ATMPs in order to understand that not all technologies can be applied to all diseases.
  - The biological mechanism of how ATMPs work in our bodies and what they change, what these therapies are for, who can benefit, and how people have already benefitted.
- Train patients on general research and development processes, similar to the European Patients' Academy on Therapeutic Innovation (EUPATI) training (44). Specifically:
  - How medicines are developed, approved and reimbursed. This includes both the European legislation on the development and approval of ATMPs as well as the national legislation to understand how local authorities make their decisions.
  - How clinical trials are performed, how evidence is collected and why it is important to collect that evidence. Sometimes patients do not have a clear perception of how long it takes to develop a new medicine, therefore it should be explained in order for them to understand why so much time is needed.
  - What are clinical trials?
  - Why is safety so important?

According to the interviewees, to be effective, education should be tailored to the situation of the patient and their interests. Considering the landscape of research on their disease, patients may have different expectations; therefore, to meet those expectations the focus of the educational path has to be wide. For example, patients can be interested because a therapy is coming to the market and they want to know how this therapy works, how it will be administered, what the outcome may be. Other patients who are waiting for a therapy that is not yet on the market may be more interested in how to get access to it before the marketing authorization process is complete. Others, affected by diseases for which there is no research ongoing, may want to know how to stimulate researchers' interest.

Moreover, there is a need to improve the dialogue between patients and clinicians, in order to consider all the possible questions related to new treatments. For patients, to have the possibility to discuss new treatments in a room with other patients and clinicians could be a great improvement and could help make them more comfortable with their decisions. The ultimate goal of a good education program should be to ensure patients properly understand the value out of these products and are in the position to make well informed decisions.

According to the interviewees, the most effective tools to train patients are webinars and face to face meetings, the latter being preferable. Face to face meetings are the preferred tool as they stimulate better exchange among participants and often better absorption of information. As a result, participants in face to face meetings are often better able to transfer their knowledge to other patients, thereby amplifying the effect of the original educational event. Training materials on the web could be an option but are considered less effective. Patient organizations can play a big role in covering these educational needs. They can organize workshops and communication campaigns about ATMPs and they have the capacity to reach a higher number of patients.

CONCLUSION AND DISCUSSION

This study reports a qualitative description on the expectation and perspectives of ten European patient advocates on ATMPs. Patients advocates recognize that the model of a limited number of expert centers administering ATMPs is the best model for effectively and safely delivering such treatments to patients. From their perspective, the success of the therapy is far more important than the location of the center administering it. In addition, that model will facilitate a centralized collection of data, which is essential to improve both the technology and to generate Real World Evidence (RWE). However, this model for administering ATMPs increases the risk of inequalities in patient access to treatment. To avoid this, ATMPs should be marketed as full-package services, including not only the drug itself but also all the necessary clinical and non-clinical services.

A major barrier preventing patient access to therapy is the very high prices ATMPs are currently marketed at. Patients are fully aware of the threat to the sustainability of health systems posed by such high prices, but this should not limit the access to life-saving treatments. Consequently, patients suggest: (1) a more transparent process for the definition of prices of ATMPs; (2) More support for academia as a possible way to develop less expensive ATMPs; (3) Centralized procurement at EU level to increase the bargaining power, especially for rare conditions and smaller countries. Price negotiation should focus on the concept of value, based on data provided by patients on the improvement of their Quality of Life, measurable benefit during clinical trials in comparison with existing therapeutic alternatives (where available) and measurement of the burden of the disease, meaning the impact of the diseases, in terms of direct and indirect costs. Patient contribution to define the value of the treatment can cover different areas:

1. Efficacy
2. Assessment of the setting, i.e., formulation of therapy
3. Impact on daily life and on Quality of Life. Patients have important insights into disease progression.

Moreover, considering the low number of patients generally included in ATMP clinical trials, it is of utmost importance to have a robust system to collect Real Word Data in long-term follow up. Patients are willing to contribute to Real Word Data collection, however the engagement and empowerment of patient communities is essential to ensure the sustainability of data collection in long-term follow up studies. In addition, to further facilitate patient engagement in the collection of follow up data, they should participate in defining Patients Reported Outcomes (PROs) and in identifying the questions most relevant to them. Patient input into ATMP development is of utmost relevance considering that these are disease modifying therapies. With the aim of accelerating access to treatment, the price negotiation process should be improved and accelerated to guarantee patients early access to innovative therapies. This acceleration despite the probable lack of robust data from clinical trials should be balanced by continuous reviewing of the price and access conditions based on the assessment of long-term Real-World Evidence. Furthermore, the risk assessment during the approval process should be reviewed, taking into account the different nature of the therapies, in particular autologous cells therapies vs. "off the shelf" products. With regard to autologous cell therapies, considering they are considered less risky for the patient, the approval process could be faster. Another key factor that should affect the timing of approval is the disease area and re-evaluating the assessment process taking into account the severity of the disease: in life threatening diseases, the level of risk that patients are willing to take is much higher than in high prevalence disease where some treatments are already available.

Concerning ethical aspects, two key messages are expressed by patients: first, all the relevant stakeholders, including patient representatives, should participate in all discussions about ethical limits and secondly, the public debate should focus more on the health benefit of these therapies. Although the patient voice is being included in ethical debates regarding genome editing (45), inclusion of patient perspectives is not yet carried out systematically. The second request from patients, to focus the ethical debate on health benefit, is especially significant as until now, the debate on ethical aspects of ATMPs has focused almost exclusively on the risk of human enhancement or on the morality

of the use of embryonic cells. Thus, a broader more balanced discussion with multiple stakeholders on the ethics of ATMPs is required (44–48). A broader, balanced, discussion on ethical aspects should be envisioned with multiple stakeholders.

One of the key aspects highlighted by the interviewees is the need to manage high and unrealistic expectations of patients. This finding is consistent with the literature on the topic (49, 50) and suggests that additional effort should be devoted to patient education on the general concepts around drug development and on the specific risks related to ATMPs. It is worth noticing that the pros and cons of every new technique are detailed, described and debated in international scientific journals, however, this information struggles to reach patients.

One of the risks which is very clearly perceived by expert patients is the spreading of non-authorized treatments that is reflected in the increasingly frequent crowdfunding requests by patients trying themselves to pay for such treatments. An approach suggested by interviewees is to educate patients on the different technologies under development, explaining their potential but also the associated risk and, finally, the different officially recognized paths to obtain access (clinical trials, compassionate use, approved drugs).

LIMITATIONS

The study was conducted on a very limited sample without any specific sampling strategy aiming at minimizing any potential sampling bias. Although the purpose of the study was to collect the view of patient advocates, this limitation in the sample may limit the validity of the results. In all cases the results presented in this paper should not be considered as the perspective of patient community as a whole. A qualitative approach was selected for this study to privilege richness of the information collected over the statistical power in representing patients' general positions. Based on the results of this preliminary qualitative study, a dedicated quantitative survey on European patients may provide more reliable data on patient perspectives on cell and gene therapies.

Moreover, from a methodological point of view, the choice of running all interviews in English may have affected the ability of interviewees to answer providing all the nuances they would have used in their mother tongue. To minimize this risk the guidelines of the interview was shared in advanced to allow interviewees to prepare and after the interview the transcript was shared again to allow them to check and potentially to adjust their statement. Nonetheless, the language barrier could not be completely overcome.

ATMP access and information vary from country to country in Europe, resulting a potential bias in opinion depending on the residency of the interviewees. However, the interviewees are expert patient advocates representing super-national patient organizations and have general visions, developments of their disease areas.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because due to their nature (semi-structured interviews) data cannot be fully anonymized. Requests to access the datasets should be directed to Stefano Benvenuti-sbenvenuti@telethon.it.

AUTHOR CONTRIBUTIONS

SBe designed the study, prepared the protocol, conducted some of the interviews, and contributed to analysing the data and to writing the manuscript. CMW contributed to writing the manuscript. SBo conduct most of the interviews, analyzed the data and contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the European Union's Horizon 2020 research and innovation program for RESTORE, under Grant Agreement No: 820292.

ACKNOWLEDGMENTS

We particularly acknowledge the contribution of Alessandra Camerini from Associazione Italiana GLUT 1 and Fabiola Maria Bertinotti for offering to pilot the guidelines for the semi-structured interviews and of all the interviewees whose contribution was fundamental for this work:

- Veerle Aertsen - EUPATI fellow, Member of IMI Pool of Patients Expert
- Simone Boselli and Karolina Hanslik – EURORDIS
- Mariette Driessens - Dutch Association of Haemophilia Patients, Member of Care and Research Working Group; Dutch Rare Disease Patients Organization – VSOP; Former member of EMA Advanced Therapies Committee
- Julian Isla Gomez – Dravet Syndrome European Federation; EMA, Member of COMP
- Paola Kruger – EUPATI fellow, EMA Expert, Member of the ISPOR Patient Roundtable
- Lydie Meheus - Anticancer Fund
- Christine Mundlos - Allianz Chronischer Seltener Erkrankungen e.V.
- Declan Noone - European Haemophilia Consortium
- Bettina Ryll - Melanoma Patients Network Europe
- Francesca Ulivi - Fondazione Italiana Diabete; Patto trasversale per la scienza.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.728529/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

SYSTEMATIC REVIEW
published: 01 December 2021
doi: 10.3389/fmed.2021.713047

ATMP Environmental Exposure Assessment in European Healthcare Settings: A Systematic Review of the Literature

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Since 2007, a new class of biologic products for human use called "advanced therapy medicinal products (ATMP)" have been legally integrated in the European Medical Agency. They consist of recombinant nucleic acid, engineered cells, cells, or tissues. In the United States, ATMP fall under the regulatory framework of biological products and the term "cell and gene therapy product" is used in the legislative and regulatory documents. Potential clinical applications are broad, particularly, in the field of cancer, inherited genetic disease, and regenerative medicine. Indeed, the benefit conferred by CD19 chimeric antigen receptor T cells led to the first engineered cell therapy products to be approved by the Food and Drug Administration (FDA) in 2017. Gene therapy products to treat orphan diseases are also extensively developed with many clinical trials ongoing in the world. Nevertheless, the use of these therapeutic products is complex and requires careful considerations in the terms of regulatory and hospital setting requirements, such as storage, handling, administration, and disposal which justify the implementation of a secured medication circuit. Through this systematic review of the literature, the authors wanted to compile data on the assessment of environmental exposure related to the use of ATMP in healthcare setting to secure their medication circuit. A literature search was conducted on PubMed and Web of Science, and 32 publications dealing with environmental exposure assessment and ATMP were selected. In addition, marketed ATMPs were identified and data regarding the environmental concerns were extracted from product information sections from European Public Assessment Reports (EPAR). The environmental contamination assessments were mainly addressed in the reviews rather than in original articles related to the use of ATMP. Most of the product information sections from EPAR suggested precautions rather than requirements when dealing with environmental consideration following ATMP handling. Nevertheless, these precautions usually remain elusive especially concerning waste disposal and the detection of biological material on the work surfaces, and mainly relate to the genetically modified organisms (GMO) over non-GMO cellular products. Pharmaceutical oversight and

OPEN ACCESS

Edited by:
Nuno M. Neves,
University of Minho, Portugal

Reviewed by:
Zhenjian Zhuo,
Guangzhou Medical University, China
Joaquim Vives,
Banc de Sang i Teixits, Spain

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *21 May 2021*
Accepted: *18 October 2021*
Published: *01 December 2021*

Citation:
Damerval M, Fagnoni-Legat C, Louvrier A, Fischer S, Limat S, Clairet A-L, Nerich V, Madelaine I and Kroemer M (2021) ATMP Environmental Exposure Assessment in European Healthcare Settings: A Systematic Review of the Literature. Front. Med. 8:713047. doi: 10.3389/fmed.2021.713047

adherence to the good preparation practices and good clinical practices are essential to ensure the safe use in term of environmental concern of these new therapeutic products in healthcare setting.

Keywords: advanced therapy medicinal products (ATMP), cell and gene therapy (CGT), environmental exposure, environmental shedding, cellular therapy, healthcare settings

INTRODUCTION

Since 2007, a new class of biological products for human use called advanced therapy medicinal products (ATMP) are legally integrated in the European Medicines Agency (EMA) (1). These innovative biotechnological products consist of recombinant nucleic acids and engineered cells or tissues which are at the origin of the complexity of their pre-clinical and clinical development, handling, regulatory framework, and classification (2). In Europe, ATMP are divided into four subcategories known as somatic cell therapy medicinal products, gene therapy medicinal products, tissue engineered products, and combined ATMP (3). In the United States (US), ATMPs also fall under the regulatory framework of biological products but only encompass two subcategories called cellular and gene therapy (CGT) products (2). Indeed, the term "CGT product" is the one used in the US legislative and regulatory documents (4). Potential clinical applications of ATMP are broad, particularly in the field of cancer, inherited genetic diseases, and regenerative medicine (5, 6). More importantly, the use of these products is rapidly expanding in the clinical settings and sometimes they are used as last resort when conventional therapeutic approaches are ineffective (7, 8).

The successful clinical transition from bench to bedside of cellular and gene therapies in the early 2000s led to the start of early phase clinical trials (9, 10). Since, several gene and gene-modified cell-based therapies are approved by the Food and Drug Administration (FDA) and EMA. Imlygic® (talimogene laherparepvec, T-VEC), a genetically modified oncolytic vector, was the second gene therapy product approved in Europe in 2016 (5, 11). Then, the clinical benefit conferred by the CD19 chimeric antigen receptor (CAR)-T cells, led to the first engineered cell therapy products to be approved by the FDA in 2017 (8, 12). Pivotal studies showed a high rate of durable responses and an increase in the global survival despite high grade toxicities (8, 13, 14). These breakthrough in the field of cancer medicine prompted to the clinical development of CAR-T cells for other hematological malignancies, such as multiple myeloma and solid tumors, such as glioblastoma despite their immunosuppressive microenvironment and technological barriers preventing T-cell entry (15, 16). Beyond their successful development in the field of immuno-oncology, ATMP are currently extensively developed in orphan diseases addressing the unmet medical needs (17, 18). Another viral vector, Zolgensma® (onasemnogene abeparvovec), developed in the orphan disease spinal muscular atrophy proved its effectiveness in terms of overall survival, motor function, motor milestone achievements, and motor unit function (19). Furthermore, the development of cellular therapy products is illustrated by the approval of several ATMP in various diseases, such as limbal stem cell deficiency with Holoclar® (*ex vivo* expanded autologous human corneal epithelial cells containing stem cells), perianal fistulas in Crohn's disease with Alofisel® (Darvadstrocel), and cartilage defect in the knee with Spherox® (spheroids of human autologous matrix associated chondrocytes) (20–22). One common point between the studies involving ATMP is the lack of information concerning the assessment of environmental exposure. The use of these therapeutic products is complex and requires careful considerations in terms of regulatory and hospital setting requirements, such as storage, handling, administration, and disposal which justify the implementation of a secured medication circuit. In the framework of ATMP, the environmental risks are described mainly as the risk of transmission of the gene modified organisms to humans other than the patient, to animals or to the environment at large (23). In Europe, the marketing authorization of ATMP falls under the mandatory scope of a central authorization procedure. Among the data submitted by the developer, an environmental risk assessment (ERA) must be present. The specific guidelines dedicated to genetically modified organism (GMO) (EMEA/CHMP/BWP/473191/2006) for both the clinical trials and marketing authorization have recently been reviewed by Whomsley R. and colleagues (24). ERA for GMO should include six steps that are: (1) the identification of characteristics which may cause adverse effects, (2) the evaluation of the potential consequences of each adverse effect if it occurs, and of the magnitude of each identified consequence, (3) the evaluation of the likelihood of the occurrence of each identified potential adverse effect, (4) the estimation of the risk posed by each identified characteristic of the GMO, (5) the application of management strategies covering the risks from the marketing of the GMO, and (6) the determination of the overall risk of the GMO. These steps should be transposable to the cell therapy medicinal products. Routes through which ATMP could come in contact with the human beings other than the intended patient, or enter the environment, include dispersal of portions of product during normal handling and use; accidental dissemination during handling and use; disposal of unused or waste medicinal product; and dispersal of GMO containing patient excreta. Once released, the GMO may spread, undergo genetic or phenotypic change, compete with existing species, infect tissue, remain latent, reproduce, transfer genetic material to other micro-organisms, transfer genetic material to

Abbreviations: ATMP, advanced therapy medicinal products; CGT, cellular and gene therapy; EMA, European Medicines Agency; EPAR, European Public Assessment Reports; FDA, Food and Drug Administration; GMO, genetically modified organisms; PCR, polymerase chain reaction; SOP, standard operation procedure; SPC, summary of product characteristics; T-VEC, Talimogene laherparep VEC.

human beings, animal, or plant species, and degrade. Despite the necessity of ERA in both the clinical trials and marketing authorization, environmental exposure assessment related to ATMP handling in healthcare setting, notably in pharmacy preparation unit dedicated or not to their manipulation, deserves to be considered. Because of the heterogeneity of ATMP, it is difficult to define the general requirements for environmental exposure assessments that are applicable to all of them, apart from a dichotomous classification between the somatic cell therapy and gene therapy medicinal products.

Through this systematic review of the literature, the authors wanted to compile the data on environmental exposure assessment related to ATMP use especially in the healthcare settings to secure their medication circuit.

MATERIALS AND METHODS

Eligibility Criteria

The population, interventions, comparison, and outcomes (PICO) model was used to formulate the questions for this study: (1) studies that considered environmental exposure assessment related to ATMP use (population), (2) studies dealing with the description of environmental exposure assessment related to ATMP use (interventions), (3) comparison criteria was not applicable, (4) studies that reported how to prevent environmental exposure in the use of ATMP and if there is a risk or not (outcomes).

Search Strategies

We searched Pubmed/Medline and Web of science databases for the studies published from January 1, 2000 to March 31, 2021. Selected keywords and Medical Subject Heading (MeSH) terms were individually selected by means of the National Library of Medicine controlled vocabulary thesaurus used for indexing articles for PubMed. The keywords and MeSH terms were combined to conduct the literature search as described in **Table 1**. This study was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (25).

TABLE 1 | The keywords and MeSH terms to conduct the literature search.

Databases	Research equations
Pubmed	• (((((((("Environmental Exposure"[Mesh]) AND "Biological Therapy"[Mesh]) NOT "Blood Patch, Epidural"[Mesh]) NOT "Blood Transfusion"[Mesh]) NOT "Cytapheresis"[Mesh]) NOT "Fecal Microbiota Transplantation"[Mesh]) NOT "Hematopoietic Stem Cell Mobilization"[Mesh]) NOT "Immunomagnetic Separation"[Mesh]) NOT "Immunomodulation"[Mesh]) NOT "Organotherapy"[Mesh] • shedding risk assessment AND gene therapy • shedding risk assessment AND cellular therapy • shedding risk assessment AND oncolytic virus • environmental shedding AND gene therapy • environmental shedding AND cellular therapy • environmental shedding AND oncolytic virus • environmental exposure AND advanced medicinal therapeutic product • safety AND advanced medicinal therapeutic product
Web of science	• TS = (risk assessment* AND gene therapy AND cell therapy product*) • TS = (environmental risk assessment* AND cell therapy product*) • TS = (environmental risk assessment* AND gene therapy*) • TS = (environmental risk assessment* AND advanced therapy medicinal product*) • TS = (environmental shedding* AND gene therapy*) • TS = (environmental shedding* AND cell therapy product*) • TS = (environmental contamination* AND gene therapy*) • TS = (health risks* AND gene therapy* AND cellular therapy product*) • TS = (risks management* AND gene therapy* AND Cellular therapy product*) • TS = (risks management* AND advanced therapy medicinal product*)

Inclusion and Exclusion Criteria

Article reporting environmental concerns related to the use of ATMP in healthcare setting written in English or French were included in this review. Exclusion criteria included translational research using the cells or animals and congress poster.

Study Selection and Data Extraction

The articles were submitted to a public reference manager (Zotero® software) to eliminate the repeated articles. Then, possible relevant articles were screened using the title and abstract by two reviewers (MK and MD) and articles that did not meet the inclusion criteria were excluded. Subsequently, the remaining full-text articles were examined by two reviewers (MK and MD). Any disagreement was resolved through discussion until a consensus was reached. The following items were extracted from each full text article that met the inclusion criteria, if available: year of publication, journal type, main location of first author, sponsor, conflicts of interest (yes, no, and not reported), type of article (original research or literature review), type of ATMP (cellular, gene therapy, and both), aim of the paper, examination of environmental exposure assessment (production, preparation, disposal), what's being watched (ATMP handling, excreta, and not reported), technique use to assess environmental exposure (PCR, sequencing, monitoring, and not reported), regulatory framework (yes or no), how to avoid environmental exposure (quarantined treated patients, disinfectants/decontamination, hygiene measures, sterilization, and not reported), and environmental risk (yes, potential, and not reported). In addition, marketed ATMP were identified and data dealing with environmental concerns were extracted from product information sections from the European Public Assessment Reports (EPAR).

Risk of Bias Assessment

Two reviewers (MK and MD) independently assessed the methodological quality of articles. The selected articles were categorized into three groups: relevant, irrelevant, and unsure.

The articles categorized as irrelevant by both the reviewers were eliminated from the study. Second, the full text of each selected article was independently analyzed by both the reviewers that make a list of articles to be included. The two list were compared, and a consensus was found in the case of disagreements between the two reviewers. When an agreement was not reached, a third reviewer made the final decision. The main reason for each article exclusion was recorded.

Additionally, the reference lists of all the selected articles were screened to identify other potentially relevant articles that were not identified by means of the electronic source. Pivotal studies of ATMP actually and previously marketed in Europe and their product information sections from EPAR were screened and analyzed.

RESULTS

Article Selection

The literature search conducted on PubMed and Web of Science identified 708 articles, among which 71 were duplicate articles and 569 were excluded after reviewing the titles and abstracts that did not match the eligibility criteria (**Figure 1**). A total of 68 articles were included for full text review, among which 42 were excluded because they were off topic, or they did not match the eligibility criteria. Two ATMP pivotal studies have been added (26, 27). Overall, 32 articles were eligible for the present systematic review.

Characteristics of Selected Articles

The characteristics of the 32 selected articles are summarized in **Table 2**. Few of them were published in the early 2000s, and there has been a considerable increase in the published articles ever since 2010 (75%, $n = 24$). Only four articles were original research, not including pivotal studies (28–31). Other were mainly state of the art or literature review.

Synthesis of the Basic Elements of Selected Article Dealing With ATMP Environmental Exposure Assessment

The synthesis of basic elements from the 32 selected articles is summarized in **Table 3**. Among the selected articles, 75% ($n = 24$) deal with gene therapy and 3% ($n = 1$) with cellular therapy. Remaining articles concerned ATMP regardless of their classification. Among the selected articles, environmental exposure assessment related to ATMP was examined during manufacturing (5%, $n = 2$), handling and manipulation (31%, $n = 12$), and waste disposal (64%, $n = 25$).

Almost half of the articles (47%, $n = 15$) involve regulatory framework, such as good manufacturing practices, good preparation practices, and/or European Union (EU) legislation.

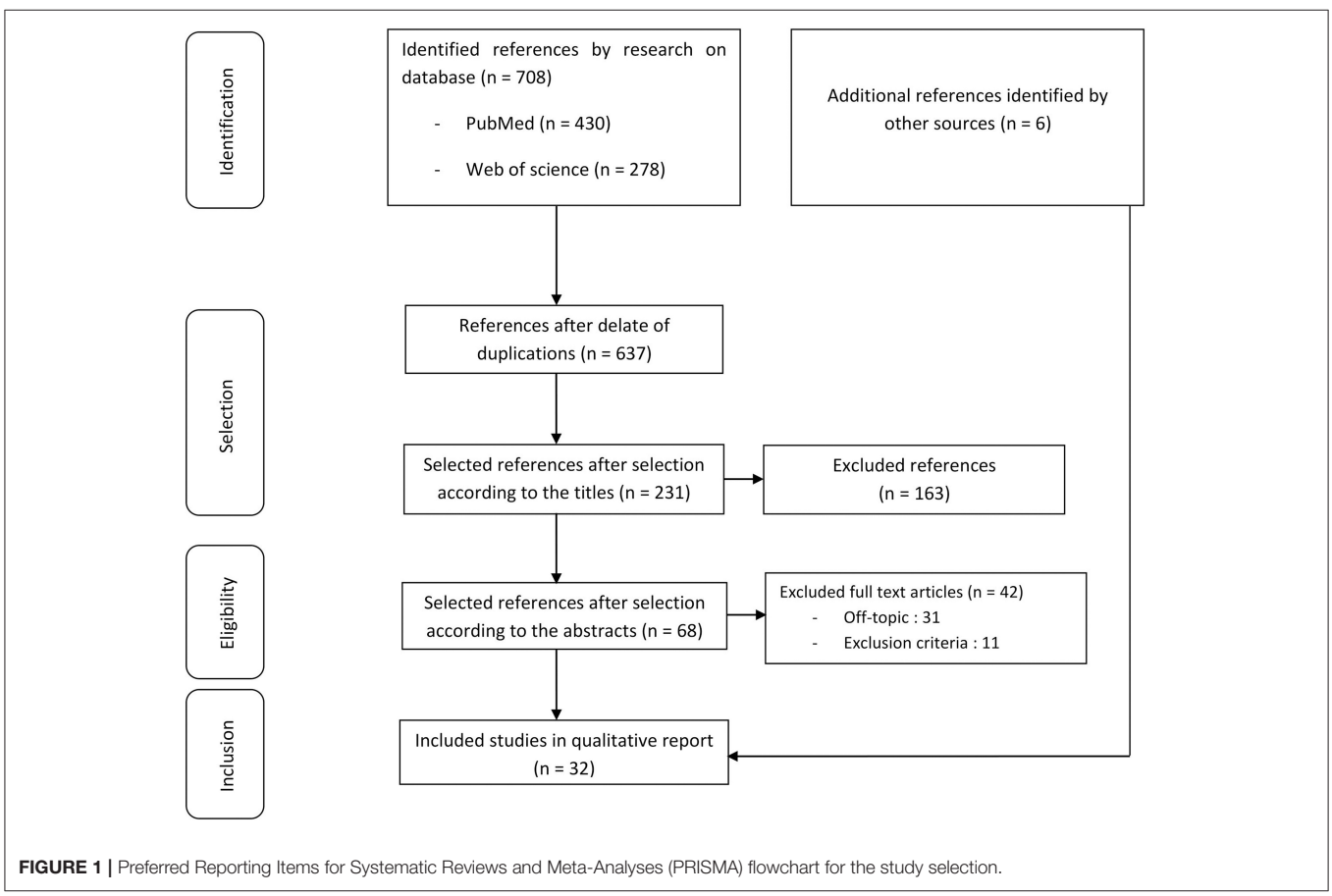

FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart for the study selection.

TABLE 2 | Synthesis of basic elements of 32 included articles.

	Number	Percentage
Years of publication		
2000–2009	8	25
2010–2019	24	75
Main location of the authors		
University	9	28
University and hospital	3	9
Hospital	9	28
Other	11	35
Original research		
Yes	6	19
No	26	81
Literature review		
Yes	26	81
No	6	19
Conflict of interest		
Yes	8	25
No	18	56
Not reported	6	19
ATMP		
Cellular	1	3
Gene therapy	24	75
Both	7	22
Examination of environmental exposure assessment		
Production	2	5
Preparation	12	31
Disposal	25	64
What's being watching		
ATMP handling	14	44
Excreta	13	41
Not reported	5	15
Technique use to assess environmental exposure		
PCR, sequencing	14	41
Monitoring	9	27
Not reported	11	32
Regulatory framework		
Yes	15	47
No	17	53
How to prevent environmental exposure		
Quarantine treated patients	8	16
Disinfectants/Decontamination	16	33
Hygiene measures/PPE	8	16
Sterilization	5	10
Not reported	12	25
Environmental risk		
Yes	7	22
Potential	20	63
Not reported	5	15

PCR, polymerase chain reaction; PPE, personal protective equipment.

PCR was used as a main technique to detect ATMP on work surfaces and the excreta or blood of a patient (26, 28, 31–33, 36, 38, 45, 46, 48, 49, 52, 54). The follow-up of a patient after treatment was approached by nine articles to evaluate the potential risk of dissemination of the ATMP (27, 34, 38, 40–44, 52). Yet, different ways of disposal were described to prevent the environmental shedding and quarantine of patients was mainly proposed ($n = 8$; 16%). Hygiene measures using the disinfectants to decontaminate and clean work surfaces and equipment as sodium hypochlorite, ethanol, or alkaline solutions following the use of ATMP were described (28–30, 33, 37–40, 45, 46, 50, 51, 54–57). Waste sterilization and especially waste autoclaving was addressed within five articles (28, 46, 54, 56, 57). Excreta, such as urine and feces were tested to assess the environmental shedding of ATMP following their administration to the patients (48, 49, 52, 54). Thus, the route of ATMP administration as well as the viral vector characteristics in case of GMO might impact the environmental shedding of ATMP.

Overall, 85% of the selected articles reported a potential environmental risk of dissemination following the use of ATMP. This risk was proven (26, 32, 34–37, 41) or considered to be potential (28–31, 38, 40, 42–49, 52–57) and concerns mainly GMO.

ATMP Product Information Sections From EPAR

Marketed ATMPs in EU are presented in **Table 4**. Among all EPAR studied (15/15), waste disposal following the local guidelines was recommended. Other information was found inconsistently, such as what to do in case of accidental exposure, the necessity to wear a personal protective equipment during ATMP handling, and the use of certain disinfectants after handling the clean work surfaces.

DISCUSSION

ATMP represent a breakthrough in the field of medicines whose active substance is produced from living tissue and demonstrate the culmination of fundamental research in biotechnology. They provide the opportunity of bringing the most innovative projects coming from translational research to the clinical setting. In line with their medication status, their management in hospital depends on the pharmacies of the hospitals (58). Their complexity and technical specificity in terms of supply, reception, storage, handling, administration, and disposal imply the creation of a dedicated medication circuit. The rapidly growing area of ATMP leads to the implementation of risk minimization measures by the pharmacists to prevent environmental and occupational exposure. This work allowed to establish a state of the art of environmental exposure assessments related to the use of ATMP in healthcare settings through the analysis of both the literature and, for ATMP with marketing authorizations in Europe, their pivotal studies, and their product information section from the EPAR. Through our literature research, 32 articles dealing with the environmental risk assessment of ATMP were selected. Among the 32 articles selected, more than three

TABLE 3 | The characteristics of the 32 selected articles.

Reference	Year	Journal	Main location of first author	0 = original research 1 = litterature review	1 = cellular therapy 2 = gene therapy 3 = both	Aims of the paper	Examination of environnemental shedding 1 = manufacturing 2 = preparation (PUI) 3 = after use	What's being watched ?	Technique use to detect	Regulatory framework	How to avoid environmental risk ?	Environmental risk
Bachtarzi et al. (32)	2019	Clinical	University	1	3	To compare control use of GMO in Europe, USA and Japan.	3	Excreta	PCR	Yes	Quarantine treated patient	Yes
Iglesias-Lopez et al. (33)	2019	Clinical	University	1	3	To summarize regulatory data about GMO in Europe and USA.	2, 3	Excreta	PCR	Yes	Physical, chemical and biological barriers	Not reported
Bubela et al. (34)	2019	Clinical	University	1	3	To review regulatory data for GMO in Canada.	3	Excreta	Environmental safety monitoring	No	Not reported	Yes
Pinturaud et al. (35)	2018	Clinical	Hospital	1	3	To identify the role of the pharmacist in the system of advanced therapy medicine.	2	ATMP handling	Not reported	Yes	Not reported	Yes
Sharpe (36)	2018	Clinical	Hospital	1	2	To review gene therapy products showing safety strategies.	3	ATMP handling	PCR	No	Not reported	Yes
Okeke et al. (37)	2017	Clinical	Hospital	1	2	To review the caracteristics of Ankara virus used as a vector.	3	ATMP handling	Not reported	Yes	Disinfectants	Yes
Renner et al. (38)	2015	Clinical	Hospital	1	3	To summarize regulatory data about GMO in Germany.	3	Excreta	PCR Environmental safety monitoring	Yes	Hygiene measures Decontamination	Potential
Montemurro et al. (39)	2015	Clinical	Hospital	1	1	To describe the italian approach concerning the arrival of the advanced medicinal products in an hospital.	1, 2	ATMP handling	Not reported	Yes	Hygiene measures Decontamination	Not reported
Lucas-Samuel et al. (40)	2015	Clinical	ANSM	1	3	To describe french regulatory data concerning advanced medicinal products.	1, 2, 3	ATMP handling	Environmental safety monitoring	Yes	Hygiene measures Decontamination	Potential
Buijs et al. (41)	2015	Clinical	Hospital & University	1	2	To review preclinical and clinical development about oncolytic viruses.	3	Excreta	Environmental safety monitoring	No	Not reported	Yes

(Continued)

TABLE 3 | Continued

Reference	Year	Journal	Main location of first author	0 = original research 1 = litterature review	1 = cellular therapy 2 = gene therapy 3 = both	Aims of the paper	Examination of environnemental shedding 1 = manufacturing 2 = preparation (PUI) 3 = after use	What's being watched ?	Technique use to detect	Regulatory framework	How to avoid environmental risk ?	Environmental risk
Narayanan et al. (42)	2014	Clinical	Institut	1	2	To review points of view of differents actors of gene therapy.	3	Excreta	Environmental safety monitoring	No	Not reported	Potential
van den Akker et al. (43)	2013	Clinical	Institut	1	2	To present the methodology to do an ERA.	3	/	Environmental safety monitoring	No	Quarantine treated patient	Potential
Hoeben et al. (44)	2013	Clinical	University	1	2	To summarize the potential biological risks of oncolytic viruses vectors.	3	/	Environmental safety monitoring	No	Quarantine treated patient	Potential
Goossens et al. (45)	2013	Clinical	Institut	1	2	To present how identify risks with Ankara viruses.	2, 3	ATMP handling	PCR	No	Hygiene measures Decontamination Quarantine treated patient Ethanol	Potential
Baldo et al. (28)	2013	Clinical	Institut	0	2	To present an ERA.	2, 3	ATMP handling	PCR, biological assay	Yes	Hygiene measures Decontamination Quarantine treated patient Autoclaving	Potential
Verheust et al. (46)	2012	Clinical	Institut	1	2	To pick up characteristics of Ankara viruses as a vector and discuss about its safety.	2, 3	Excreta	PCR, biological assay	Yes	Cleaning up the skin with alcohol 70 % Hygiene measures Chemical decontamination, steam sterilization Quarantine treated patient	Potential
Koppers-Lalic and Hoeben (47)	2011	Clinical	University	1	2	To classify viruses according to their environmental impact.	3	/	Not reported	No	Not reported	Potential

(Continued)

TABLE 3 | Continued

Reference	Year	Journal	Main location of first author	0 = original research 1 = litterature review	1 = cellular therapy 2 = gene therapy 3 = both	Aims of the paper	Examination of environnemental shedding 1 = manufacturing 2 = preparation (PUI) 3 = after use	What's being watched ?	Technique use to detect	Regulatory framework	How to avoid environmental risk ?	Environmental risk
Tiesjema et al. (48)	2010	Clinical	Institut	1	2	To present shedding data of viral vector according to the route of administration.	3	Excreta	PCR, southern blot, ELISA, transgene expression, infectious assay	No	Not reported	Potential
Brandon et al. (49)	2010	Clinical	Institut	1	2	To present shedding data of viral vector according to the route of administration.	3	Excreta	PCR, southern blot, ELISA, transgene expression, infectious assay	No	Not reported	Potential
Anliker et al. (29)	2010	Clinical	Institut	0	2	How do an ERA ?	3	ATMP handling	Not reported	Yes	Hygiene measures Decontamination Quarantine treated patient	Potential
Pauwels et al. (50)	2009	Clinical	Institut	1	2	To review how to improve the safety with the use of Lentivirus vector.	2	ATMP handling	Not reported	No	Hygiene measures Decontamination Disinfectants PPE	Not reported
Kuhler et al. (51)	2009	Clinical	Medical product agency	1	3	To discuss about environmental impact of biological medicinal products.	3	ATMP handling	Not reported	Yes	Disinfectants	Not reported
Schenk-Braat et al. (52)	2007	Clinical	Hospital	1	2	To review studies about gene therapy and environmental impact.	3	Excreta	PCR, biological assay ELISA Environmental safety monitoring	No	Not reported	Potential

(Continued)

TABLE 3 | Continued

Reference	Year	Journal	Main location of first author	0 = original research 1 = litterature review	1 = cellular therapy 2 = gene therapy 3 = both	Aims of the paper	Examination of environnemental shedding 1 = manufacturing 2 = preparation (PUI) 3 = after use	What's being watched ?	Technique use to detect	Regulatory framework	How to avoid environmental risk ?	Environmental risk
Bleijs et al. (53)	2007	Clinical	University	1	2	To summarize regulatory data concerning gene therapy in the Netherlands.	3	/	Not reported	Yes	Quarantine treated patient	Potential
Moss et al. (31)	2004	Clinical	University	0	2	Clinical study about safety of a viral vector used in cystic fibrosis.	3	Excreta	PCR	No	Not reported	Potential
Tenenbaum et al. (54)	2003	Clinical	Hospital & University	1	2	To review characteristics of two viral vectors.	3	Excreta	PCR	No	Cleaning up materials with alkaline solutions with a pH greater than 9 or by autoclaving	Potential
Gaudet et al. (26)	2013	Clinical	University & Hospital	0	2	To show the efficacy and tolerability of the product.	3	Excreta	PCR	No	Not reported	Yes
Thompson et al. (27)	2018	Clinical	Hospital	0	2	To show the efficacy of the product.	3	/	Environmental safety monitoring	No	Not reported	Not reported
McBride et al. (30)	2018	Clinical	University	0	2	To provide an overview of the preparation and handling of imlygic.	2	ATMP handling	PCR	Yes	PPE Occlusive bandage Disinfectants: bleach, isopropanol	Potential
Petrich et al. (55)	2020	Pharmaceutical	Hospital	1	2	To provide a comprehensive review of gene therapy.	2	ATMP handlig	Not reported	No	Disinfectants	Potential
Stoner et al. (56)	2003	Pharmaceutical	Hospital	1	2	To illustrate the development of procedures to minimize risks to health, patient safety and the environment.	2	ATMP handling	Not reported	Yes	Disinfectants PPE Decontamination Autoclaving, incineration Occlusive bandage	Potential
Vulto et al. (57)	2007	Pharmaceutical	University	1	2	To specify the requirements of each step for the gene therapy drug circuit.	2	ATMP handling	Not reported	Yes	PPE Decontamination, Disinfectants Autoclaving, inactivation	Potential

TABLE 4 | Environmental exposure assessment consideration from the European Public Assessment Reports (EPAR) of advanced therapy medicinal products (ATMP) in Europe.

	Name	Pharmaceutical form	Route of administration	General description	Therapeutic indication	Approved by/date	Stopped	Environmental exposure assessment consideration
Cell therapy medicinal products	Alofisel®	Suspension for injection	Intralesional use	Human adipose tissue-derived MSCs	Complex perianal fistulas in CD	EMA 2018 March		Local requirements
	Chondrocelect®	Implantation suspension	Implantation	Autologous cell therapy based on chondrocytes	Cartilage defects	EMA 2009 October	2016 July	Local requirements
	MACI®	Implantation matrix	Implantation	Cultured chondrocytes on a porcine type I/III collagen membrane	Single or multiple symptomatic full-thickness cartilage defects of the knee with or without bone involvement in adults	EMA 2013 June	2014 September	Local requirements
	Provenge®	Dispersion for infusion	Intravenous use	PBMNCs (primarily DCs) activated with PAP and GM-CSF	Asymptomatic or minimally symptomatic metastatic castrate resistant (hormone refractory) PCA	EMA 2010 September	2015 May	Local requirements Aseptic handling Potential transmission
	Holoclar®	Living tissue equivalent	Implantation	HCEpC containing stem cells	Severe limbal stem cell deficiency	EMA 2015 February		Any unused medicinal product or waste material must be returned to the manufacturer.
Gene therapy medicinal products	Glybera®	Solution for injection	Intramuscular use	In vivo AAV-based gene therapy	Lipoprotein lipase deficiency	EMA 2012 October	2017 October	Local requirements Virucidal disinfectant
	Imlygic®	Solution for injection	Intralesional use	Live, attenuated HSV-1 genetically modified to express hGM-CSF	Unresectable cutaneous, subcutaneous, and nodal lesions in recurrent melanoma after initial surgery	EMA 2015 December		Local requirements PPE Accidental exposure Occlusive bandage
	Luxturna®	Solution for injection	Subretinal use	Live, non-replicating AAV2 genetically modified to express hRPE65 gene	Biallelic RPE65 mutation-associated retinal dystrophy	EMA 2018 November		Local requirements PPE Virucidal disinfectant Accidental exposure
	Kymriah®	Dispersion for infusion	Intravenous use	CD19- targeted genetically modified T-lymphocytes	Patients up to 25 years of age with refractory B-ALL, who are in relapse post-transplantation or in second or later relapse, and adult patients with r/r DLBCL after two or more lines of systemic therapy	EMA 2018 August		Local requirements PPE

(Continued)

BRINGING ADVANCED THERAPY MEDICINAL PRODUCTS (ATMPS) TO THE CLINIC AND BEYOND: HOW TO ENSURE THE SUSTAINABLE AND AFFORDABLE INTRODUCTION OF ATMPS INTO HEALTHCARE

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Citation: Volk, H.-D., Griscelli, A. B., Battaglia, M., Neves, N. M., eds. (2022). Bringing Advanced Therapy Medicinal Products (ATMPs) to the Clinic and Beyond: How to Ensure the Sustainable and Affordable Introduction of ATMPs Into Healthcare. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-780-3

quarter were published over the last 10 years, demonstrating the rapidly growing area of ATMP.

Two articles addressed the manufacturing step of ATMP in terms of regulatory framework, manufacturing, and quality control guidelines. The manufacturing step mostly focused on environmental concern related to the prevention of cross contamination and the establishment of process and standard operating procedures (SOP) to maintain a clean working environment to protect the ATMP. However, the measures taken to protect the ATMP indirectly apply to protect the environment. Twelve selected articles addressed the preparation steps of the ATMP in healthcare setting. Hygiene measures and decontamination were systematically mentioned with, among others, the use of bactericidal or virucidal agents to prevent environmental shedding. In an original article of McBride, T-VEC handling was presented (30). To prevent environmental shedding, the authors recommended the use of personal protective equipment during T-VEC preparation and administration and the use of disinfectants to clean work and room surfaces exposed to T-VEC. Similar recommendations were made by Stoner N et al. and Pietrich J et al. (55, 56). Nevertheless, the realization of a dedicated test, such as PCR to assess the presence of the GMO following its utilization and cleaning was not suggested. In 2007, Vulto AG et al. published general guidance about gene therapy handling within hospital pharmacy and suggested similar precautions should be taken in the handling of gene medicine and cytotoxic agents, especially concerning the prevention of cross-contamination (57). Unlike antineoplastic drugs, the existence of dedicated kits to assess environmental contamination on the work surfaces were not mentioned. In a study realized by Moss RB et al. that aimed to assess the safety and efficacy of a viral vector for the treatment of cystic fibrosis, the authors analyzed excreta of the patients using PCR assays to assess environmental shedding of the virus (31). Despite detection of the virus in the sputum samples of a patient, no minimal recommendations were proposed by the authors to prevent environmental shedding. Similar observations were related in the study of Baldo *et al.*, which deals with gene therapy having a potential risk of dissemination depending on the vector used (28). According to the authors, it is important to analyze environmental shedding with regards to the capacity of the virus to replicate and resist within "the environment" and to quarantine the treated patients, if necessary. Environmental shedding may also depend on the route of administration of the ATMP. Indeed, Tiesjema et al. indicated that the routes of shedding for HAdV-5 depend on the route of administration (48). In the Glybera® 's (alipogene tiparvovec) pivotal study, the authors specified that following its administration, the treatment may result in a low risk of dissemination in the environment (26). Indeed, the genetically modified viruses were detected in blood, urine, and saliva of the treated patients by qualitative PCR until several weeks following the ATMP injections. Nevertheless, no recommendations regarding the management of these excreta were formalized by the authors. No information concerning storage, handling, the detection of ATMP on the work surfaces and waste disposal were mentioned in original articles from our literature search except for T-VEC (30). As previously described, original research articles mainly deal with investigational medicinal product safety and efficacy.

Most of product information sections from EPAR suggest that specific precautions should be taken regarding environmental consideration following ATMP handling. Thus, in a section entitled "clinical particular," the summary of product characteristics (SPC) of tisagenlecleucel indicates the precautions that might be taken by the healthcare professionals before handling or administering the medicinal product to prevent transmission. The precautions to be taken during transport and for wastes disposal are also mentioned in a section entitled "Special precautions for disposal and other handling." Thus, tisagenlecleucel "should be transported within the facility in closed, break-proof, and leak-proof containers." Waste disposal is not much discussed. Yet, it is advisable to follow the local guidelines for biological wastes disposal. Concerning axicabtagene ciloleucel, the precautions suggested were identical. Overall, there are dedicated precautions for the disposal and handling of CAR-T cells products in the SPC but no information about the risk of surface contamination and product detection were specified. In the T-VEC section "special precautions for disposal and other handling," recommendations concerning handling and administration, personal protective equipment, accidental spills, and waste disposal were specified. The same recommendations were specified in the product section information for Luxturna® (voretigene neparvovec), onasemnogene abeparvovec, Zolgensma®, Provenge® (Sipuleucel-T), Zalmoxis®, and Strimvelis®. Nevertheless, information still remains elusive especially concerning waste disposal and the detection of medicinal product on work surfaces. Finally, we noticed that the procedure to follow in case of accidental exposure was also detailed in the same section referring to use virucidal agent in case of spill(s). As far as cell therapy is concerned, the environmental exposure assessment is once again a poorly discussed subject. Yet, among cell therapy products, only the SPC of Chondrocelect® specifies, without any further details, that any drug or waste material must be disposed of in accordance with the current regulations.

Management of ATMP is complex, preventing the establishment of a single standardized pharmaceutical circuit for all of them. Furthermore, their specific storage and preparation as well as their classification as GMO or not determine how they need to be handled. Regarding GMO, the assessment of the probability that a potential hazard occurs that determines the level of risk. The level of risk then allows to determine ways to control them to ensure the protection of humans and the environment. As discussed above, antineoplastic drug handling implies a strict aseptic process to prevent cross-contamination within the pharmacy preparation units. Indeed, the experience of pharmacists, justified by the centralization of the reconstitution of antineoplastic drugs, provides an adequate basis for the handling of ATMP in health settings (55, 58, 59). Environmental concern regarding the use ATMP and not only GMO, as previously described, require adaptation in the pharmacies in terms of facilities, equipment, SOP, and waste disposal (57). Storage and manipulation of ATMP need to be performed in a dedicated area. The manipulation of GMO must be conducted

in contained cabinet or isolator in negative pressure relative to the pressure of the immediate environment to protect the worker and the environment as well as the product itself. A dedicated high-efficiency particulate air (HEPA) filtering of the extracted air to protect the environment and input air to protect the product is necessary in area of both GMO and non-GMO ATMP manipulation. The establishment of SOP for storage, cleaning, preparation, personal protective clothing dedicated to preparation and administration, transport, accidental exposure, disinfection and decontamination, and disposal of waste is a minimum requirement to prevent environmental shedding. Obviously, these SOPs might be interconnected. Thus, whatever the ATMP, and considering the GMO risk group, disposable personal protective clothing, handling, and administration equipment directly in contact with the ATMP should be autoclaved (sterilization at 134°C during 20–30 min in air saturated with water vapor) if possible, using appropriate sealed container and then incinerated. Non-disposable equipment and material should be cleaned according to institutional SOP and manufacturer instruction to prevent environmental shedding. As mentioned above, the instructions present within EPAR, when they exist, always remain elusive. However, because of their diversity, GMO and especially viruses may have heterogeneous sensitivity to liquid chemical disinfectants. In that context, the recombinant associated viruses that are already used as gene delivery vehicles for approved ATMP have been described as the good virus models for testing the virucidal efficacy of disinfectants. Two studies evaluating the chemical sensitivity of different human adenovirus serotypes have concluded that the inactivation method varies according to each virus serotype demonstrating the need for knowledge and thus providing clear instructions for inactivation methods suitable for each ATMP using a viral vector (60, 61). Additionally, both studies demonstrated that complete inactivation using suitable disinfectants can be done safely and quickly.

This manuscript has several deficiencies. The articles included were limited to English and French only. In addition, the abstract or meeting articles as well as congress posters were excluded although their scientific contribution could have been taken into consideration. This systematic review has not been registered online. Bias due to selective non-reporting (or incomplete reporting) that were measured and analyzed by the trial investigators are likely not to be disclosed. This literature review and data collected from EPAR are biased to published data that may not reflect the actual knowledge on the environmental impact of ATMP. Beyond existing undisclosed data, key considerations, such as dispersal of GMO from patient excreta in the clinical trials may not be known or planned in clinical trial development.

CONCLUSION

Many challenges remain to be fulfilled in environmental contamination assessment related to the use of ATMP within the pharmacy preparation units, healthcare settings, and beyond. Because the use of these new treatments is a rapidly expanding field with increasing use in the clinical trials and routine practice, the guidelines are eagerly awaited. Even though environmental contamination assessment is poorly addressed in original articles related to the use of ATMP, most of the product information sections from EPAR suggested precautions rather than requirements when dealing with environmental consideration following ATMP handling. Nevertheless, information usually remains elusive especially concerning waste disposal and the detection of biological material on work surfaces, and mainly relate to the GMO than non-GMO cellular products. Pharmaceutical oversight and adherence to good preparation practices and good clinical practices are essential to ensure the safe use of these new therapeutics in healthcare setting in term of environmental concern. Additionally, this work demonstrates the necessity to adopt a multidisciplinary approach involving the clinicians, nurses, pharmacists, and biologists to assess and control environmental exposure to ATMP in the healthcare settings at all steps, from their reception to their administration, and suggest the importance to monitor excreta of a patient during the clinical trials to define recommendations to prevent environmental shedding following their use.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MK and IM: Conceptualization. MK and AL: Methodology. MD, CF-L, and MK: Writing. MK, MD, and AL: Literature search. MD, CF-L, AL, SF, A-LC, SL, and VN: Critically revised work. MK: Supervision. All authors were involved in the design, data collection, analysis, and manuscript preparation.

FUNDING

This work was supported by the Region Bourgogne Franche-Comté (PERSONALISE, subvention #2019-0077).

ACKNOWLEDGMENTS

We thank Mihail Turcanu and Camille Hosotte for English writing assistance.

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frontiers in Medicine

ORIGINAL RESEARCH
published: 10 December 2021
doi: 10.3389/fmed.2021.729018

Evidence of Antitumor and Antimetastatic Potential of Induced Pluripotent Stem Cell-Based Vaccines in Cancer Immunotherapy

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OPEN ACCESS

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: 22 June 2021
Accepted: 18 November 2021
Published: 10 December 2021

Citation:
Kishi M, Asgarova A, Desterke C, Chaker D, Artus J, Turhan AG, Bennaceur-Griscelli A and Griscelli F (2021) Evidence of Antitumor and Antimetastatic Potential of Induced Pluripotent Stem Cell-Based Vaccines in Cancer Immunotherapy. Front. Med. 8:729018. doi: 10.3389/fmed.2021.729018

Cancer is maintained by the activity of a rare population of self-renewing "cancer stem cells" (CSCs), which are resistant to conventional therapies. CSCs over-express several proteins shared with induced pluripotent stem cells (iPSCs). We show here that allogenic or autologous murine iPSCs, combined with a histone deacetylase inhibitor (HDACi), are able to elicit major anti-tumor responses in a highly aggressive triple-negative breast cancer, as a relevant cancer stemness model. This immunotherapy strategy was effective in preventing tumor establishment and efficiently targeted CSCs by inducing extensive modifications of the tumor microenvironment. The anti-tumoral effect was correlated with the generation of CD4+, CD8+ T cells, and CD44+ CD62L- CCR7low CD127low T-effector memory cells, and the reduction of CD4+ CD25+FoxP3+ Tregs, $Arg1^+$ CD11b+ Gr1+, and $Arg1^+$ and CD11b+ Ly6+ myeloid-derived suppressor cell populations within the tumor. The anti-tumoral effect was associated with a reduction in metastatic dissemination and an improvement in the survival rate. These results demonstrate for the first time the clinical relevance of using an off-the-shelf allogeneic iPSC-based vaccine combined with an HDACi as a novel pan-cancer anti-cancer immunotherapy strategy against aggressive tumors harboring stemness features with high metastatic potential.

Keywords: breast cancer, CSC, vaccine, iPSCs, HDACi

INTRODUCTION

During the last decade, the concept of tumor heterogeneity has been extensively explored in solid tumors, leading to the identification, in several types of cancers, of dedifferentiated cancer cells, designated as "tumor-initiating cells" or "cancer stem cells" (CSCs) (1, 2). Within the bulk of a tumor, these cells represent a minor population with defined functional and molecular characteristics: (i) they are able to reinitiate tumor growth in immunodeficient mice by self-renewal capacity (3, 4), (ii) they exhibit various degrees of stemness signature based on transcriptomic and epigenetic molecular profile (5–7). Specifically, current evidence indicates that in addition to the

well-known oncoprotein c-MYC, some of the key regulators of embryonic stem cells (ESCs), such as OCT4, SOX2, and NANOG, are also abnormally over-expressed in CSCs of a broad range of malignancies (7, 8). These three factors participate in a highly integrated network along with c-MYC and polycomb proteins that use the epigenetic machinery to remodel chromatin through histone modification and DNA methylation. This ability to induce major epigenetic modifications was first demonstrated by groundbreaking experiments leading to the discovery of induced pluripotent stem cell (iPSC) technology (9, 10). iPSCs are closely linked to ESCs, as both express the same auto-regulatory circuitries (11). Subsequent analyses of the genomic characteristics of iPSCs revealed the occurrence of genetic abnormalities in iPSC arising either from the initial somatic parental cell (12, 13) or during their expansion *in vitro* (12).

Previous studies have explored ESC/iPSC-based cancer vaccines as source of immunogenic tumor associated antigens (TAA) (14–18). However, they could not assess their effect on metastatic spread because the model cell line used in these studies lacked metastatic potential. Given these considerations, we used a cancer stemness model to explore an iPSC-based vaccination strategy exploring the possibility to inhibit metastatic dissemination. To this purpose, we have used an aggressive murine cancer cell line generating lung metastasis after implantation. In addition, as cancer stemness is strongly associated with immunosuppressive genes that inhibit T cell activation, we have combined the iPSC vaccination along with an epigenetic modification using HDAC inhibitors. It is indeed known that tumor microenvironment (TME) represents a privileged niche in which diversification of tumor clones can occur (19). It is also well-documented that this immunosuppressive niche contributes to the establishment, progression, and immune escape in various types of cancers. This niche is generated through epigenetic alterations capable of efficiently and accurately reprogramming the TME. The latter was shown to implicate several mechanisms including DNA methylation, histone post-translational modifications, and non-coding RNA-mediated regulation (20). Molecules such as histone deacetylase inhibitors (HDACi) are currently under evaluation to modify this immunosuppressive TME able to convert a tumor from an immune suppressive (cold) to an immune permissive (hot) niche (21). In a therapeutic point of view, the short-chain fatty antiepileptic drug Valproic Acid (VPA) is a class I selective HDACi, acting with IC50 values ranging from 0.4 to 3 mM, was shown to be able to modify TME by decreasing in particular myeloid-derived suppressor cells (MDSCs) (22) without significant side effects (23).

We show here that iPSCs-based vaccine combined with VPA in a metastatic model of aggressive murine cancer cell line, prevent the establishment of CSCs-enriched tumors and to inhibit efficiently the development of lung metastases.

MATERIALS AND METHODS

Cell Line Isolation and Maintenance

Primary fibroblasts from BALB/c mice were reprogrammed into pluripotency by ectopic expression of OCT4, SOX2, c-MYC, and KLF4 using a Cre-Excisable Constitutive Polycistronic Lentivirus (EF1alpha-STEMCCA-LoxP backbone from Millipore). A C57BL/6-derived iPS cell line was purchased from ALSTEM (Richmond, CA). Both murine iPSC lines were maintained on mitomycin-treated MEFs in DMEM glutamax (Gibco), 15% fetal bovine serum (Eurobio), 1% penicillin and streptomycin (Invitrogen), 1 mM 2-mercaptoethanol (Sigma), and 1,000 units/mL of Leukemia Inhibitory Factor. Pluripotency was confirmed by FACS analysis and Teratoma assays in immunodeficient mice (NOD-SCID) after injection of 2×10^6 iPSC per mouse. Two months later, the presence of three germline layers were confirmed in teratomas by histology and Immunohistochemistry (IHC) as described (24). The vaccine batch was prepared from miPSC cultured on gelatin (Sigma) and Essential 8 medium (Thermo Fisher Scientific). miPSCs were incubated for 24 h with 0.5 mM of VPA, known to improve the induction of bona fide iPSCs and to avoid iPSCs senescence (25, 26) followed by a lethal irradiation at 15 Gy. The breast cancer line 4T1 was obtained from ATCC (CRL-2539). 4T1 MammoSpheres (MSs) were produced in 9 days in low-attachment 6-well-plates at density of 100,000 cells per well in MEF-conditioned medium (3/4 MEF-conditioned medium + 1/4 mES medium + 4 ng/mL bFGF), and addition of TNF-alpha (20 ng/mL), and TGF-β 1 (10 ng/mL) (Cell Signaling Technology). 4T1 cell line was transduced by the retroviral vector pMEGIX encoding the genes for firefly luciferase. Stable clones of 4T-luc were isolated and selected by bioluminescence imaging systems based on luciferase expression.

Transcriptome Meta-Analysis of 4T1 Cells and miPSCs

To confirm the stemness signature of murine 4T1, we have performed transcriptome microarray analysis in context of *in vitro* and *in vivo* tumor experiments (Clariom™ S Assay, mouse kit, Thermofisher scientific France) according to the manufacturer's instructions. Our 4T1 *in vitro* and 4T1 *in vivo* transplant transcriptome experiments were integrated with mammary gland samples from GEO dataset GSE14202 and mouse iPSC data from GEO dataset GSE15267. Cross batch normalization was applied with Combat function from SVA R bioconductor package (27). LIMMA analysis was performed between 4T1-transplant samples vs. in 4T1 *in vitro* samples to identify differential expressed genes (28). Expression heatmap was realized with pheatmap R-package and unsupervised principal component analysis plot ggfortify R package post prcomp R function. Functional enrichment was performed with Toppgene web application (29) on GO-BP, MSigDb and DisGeNET (30) databases. Functional enrichment network was built with Cytoscape application version 3.6.0 (31).

Transcriptome Analysis of 4T1 Cells Treated With Valproic Acid

Total RNA was extracted following the instructions of the manufacturer (TRIzol, Life Technologies) from 4T1 cells treated with and without valproic acid (VPA) at dose of 0.5 mM for 10 days. Microarray probes were synthetized in

one cycle of RNA amplification in which molecules were labeled (Affymetrix microarray station, Affymetrix, CA). The labeled microarray probes were hybridized on a Mouse Clariom S (mm10) microarray (Thermo Fisher Scientific), and the CEL files of microarray data obtained from the Affymetrix platform were normalized using the RMA method in Affymetrix Expression Console software (Affymetrix, CA). Gene-set enrichment analysis was performed with the online java module of GSEA software, version 3.0, while a network-based gene-set enrichment analysis was performed with Cytoscape software, version 3.6.0. Bioinformatics analyses were performed in R version 3.4.1; the R-package made4 was used to create an expression heatmap using Euclidean distances and the Ward method, and the FactoMineR R-package was used to perform an unsupervised principal component analysis. Genes with significant differences in expression were selected with the SAM algorithm using a FDR threshold of 5 percent.

Animal Model

Wild-type female BALB/c mice, 8–10 weeks old, were purchased from Janvier Laboratory. Protocols of animal experiments were approved by the Animal Care Committee of the Val de Marne. *In vivo* studies were designed using 4 to 10 BALB/c mice in each group. Treatment consisted of two subcutaneous injections (1-week interval between injections) of 2×10^6 miPSCs. One week following the second injection, mice were inoculated with 5×10^4 4T1Luc cells, directly transplanted into mammary fat-pad following by 15 days of VPA treatment, orally administered at dose of 4 mg/mL, an approach that leads to approximate concentrations of 0.4 mM VPA in plasma, as previously reported (32). Tumor growth was followed *in vivo* by bioluminescence imaging using the IVIS Spectrum system and quantified by the Living Image software (Perkin Elmer). Lungs were incubated *in vitro* with 150 μg/mL luciferin to quantify metastatic tumors. Tumors size and volume was measured and dissociated by GentleMACS dissociator (Miltenyi Biotec) without enzyme R (Tumor dissociation kit, Miltenyi Biotec). Spleens were dissociated using a cell strainer (Fisher), after removing red blood cells (RBC Lysis Buffer, eBioscience).

Staining of Immune Cells and Tumor Cells for FACS Analysis

Splenocyte and immune cells from tumor micro-environment were analyzed by FACS for the expression of CD44, CD24, CD45, CD8a, CD25, CD279 (PD-1), MHC class I, CD3, CD11b, Gr-1, CD4, Ly-6C, CXCR5, CD22, CD62L, Arginase 1/ARG1, CD197, CD127 (**Supplementary Table 1**). Dead cells were excluded using 7-aminoactinomycin D (7-AAD) and zombie violet. Immune stimulation was performed using 50 ng/mL Phorbol 12-Myristate 13-Acetate (PMA) (Sigma) and 500 ng/mL ionomycin b (Sigma) in RPMI 1640 medium (Gibco). FACS analysis was conducted using MACSQuant analyzer (Miltenyi Biotec). The proportion of T-cell subpopulations has been reported among total CD3 + lymphocytes in analysis of blood samples. The proportion of cells in tumor samples and spleen, was reported among the total CD45 + hematopoietic cell population.

Aldefluor Assay

The Aldefluor kit (Stem Cell Technologies) was used to characterize the ALDH activity on 4T1 cells as described (33). Cells were incubated at 37°C in Aldefluor assay buffer, which contained the ALDH substrate BODIPY-aminoacetaldehyde. To determine baseline fluorescence, the enzymatic activity of ALDH was blocked by the inhibitor DEAB. FACS analysis was performed using a MACSQuant analyzer (Miltenyi Biotec).

Transcriptome Analysis of 4T1 Tumors From Mice Vaccinated With miPSCs and VPA

Total RNA was extracted from 4T1 tumors in treated and control mice, following the instructions of the manufacturer (TRIzol, Life Technologies). Microarray probes was synthetized by one cycle of RNA amplification in which molecules were labeled in an Affymetrix microarray station (Affymetrix, CA). Labeled microarray probes were hybridized on a Mouse Clariom S (mm10) microarray (Thermo Fisher Scientific). The CEL files of microarray data obtained from the Affymetrix platform were normalized using the RMA method in Affymetrix Expression Console software (Affymetrix, CA) (34). As described by Lyons et al. (35), gene modules of specific immune cell expression profiles were constructed for the purpose of immune-cell-specific profiling of cancer. With these specific immune modules, we conducted gene-set enrichment analyses using the online java module of GSEA software version 3.0 (36). A network-based analysis of gene enrichment was performed with Cytoscape software version 3.6.0 (31). Differentially expressed genes were identified with a rank products analysis. A functional analysis of genes that were upregulated in the vaccinated group was performed using the Gene Ontology biological process database and the DAVID application from the NIH website (37).

qRT-PCR

Total RNA was isolated using TRIzol Reagent (Life Technologies) and reverse transcription was performed using MultiScribe Reverse Transcriptase (Applied Biosystems) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed in duplicate using SYBR Green PCR Master Mix (Applied Biosystems) on an Agilent Technologies Stratagene MX3005p apparatus. The expression levels of genes were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of the primers used are: for CXCL9: Fw: CCATGAAGTCCGCTGTTCTT, Rv: TGAGGGATTTGTAGTGGATCG, for CXCL10: Fw: ATCAGCACCATGAACCCAAG, Rv: TTCCCTATGGCCCTCATTCT, for CXCL13: Fw: ATGAGGCTCAGCACAGCA, Rv: ATGGGCTTCCAGAATACCG, for GAPDH: Fw: GAGAGGCCCTATCCCAACTC, Rv: TCAAGAGAGTAGGGAGGGCT.

Quantification and Statistical Analyses

All values are expressed as mean ± s.e. Differences among groups were assessed, as appropriate, using either an unpaired two-tailed Student's *t*-test or a one-way/two-way analysis of variance (ANOVA) in PRISM GraphPad software or Microsoft Office Excel software. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RESULTS

Poorly Differentiated Murine 4T1 Breast Tumors Display an IPSCs-Like Expression and Stemness Signature

Cross batch normalization, transcriptome integration of 4T1 *in vitro* and 4T1-transplant samples with samples of mouse iPSCs and mouse micro dissected mammary gland (**Figure 1A**) have allowed to perform a differential expression analysis that was performed with LIMMA algorithm between 4T1-transplant and 4T1 *in vitro* (**Figure 1B**). This supervised analysis allowed to identify 325 up regulated genes in 4T1-transplant condition (Log_2 Fold Change >2 and Adjusted $p < 0.01$, **Supplementary Table 2**). Unsupervised clustering performed with up regulated genes in 4T1-transplant allow to aggregate miPSCs samples with 4T1 transplant samples in one cluster and 4T1 *in vitro* samples with micro-dissected mammary gland samples in another cluster (Euclidean distance and ward.D2 method, **Figure 1C**). Functional enrichment analysis performed with these 325 up regulated genes in 4T1-transplant on DisGeNET database allowed to identify a main enrichment in triple negative breast cancer disease (**Figure 1D**). Triple negative breast cancer enriched signature in 4T1-transplant condition allowed to build a large molecular related network (**Figure 1E**). In addition, enrichment on GO-BP database allowed enrichment of terms in relation with development and morphogenesis and especially genes implicated in epithelial and tube development which are components important for mammary gland pathophysiology (**Supplementary Figures 1A,B**). Functional enrichment performed on MSigDb version 7.3 allowed also to identify an enriched molecular network implicating relations with microenvironment such as: focal adhesion kinases, integrin and extracellular matrix organization with several collagens and metalloproteinases (**Supplementary Figures 1C,D**) as well in cell mobility and migration (**Supplementary Figures 2A,B**). All these functional enrichments are in agreement with an invasive process and with a mesenchymal characteristic of this TNBC mouse model after injection in mice.

We then used FACS analysis to quantify the expression of the breast cancer stem cell markers CD44 and CD24 (38) in 4T1 cells recovered *in vitro* and *in vivo* 12 and 28 days after injection into mammary fat pads. A population of $CD44^{+/high}/CD24^{-/low}$ cells was identified in 4T1 cells *in vivo*, with a frequency up to 32% (**Supplementary Figure 3**). Since it is well-known (38) that induction of an EMT in transformed mammary epithelial cells yields cells with $CD44^{high}/CD24^{low}$ antigenic phenotype, we wished to confirm the presence of EMT markers by whole transcriptome unsupervised principal component analysis.

Valproic Acid Modify the Transcriptomic Landscape of 4T1 Cells

Before testing the *in vivo* immune-modulatory effect of VPA in combination with iPSC-based vaccine, we asked whether VPA could modulate on its own, the immune-related gene expression in 4T1 cells. To this purpose, we performed a transcriptome analysis on 4T1 cells treated *in vitro* with 0.5 mM of VPA for 10 days, and compared this to the transcriptome of untreated cells. These analyses identified 117 immune-related genes implicated in TNF alpha and/or IFN-alpha and IFN-gamma signaling (**Figure 2A**). These results were confirmed by a gene-set enrichment analysis that revealed significant enrichment in these three immune gene sets (**Figure 2B**). In addition, using the SAM algorithm we were able to identify 44 immune-related genes that demonstrated expression differences between the VPA-treated samples and their control counterparts (**Figure 2C**, **Supplementary Table 3**). These were validated by principal component analysis (**Figure 2D**, $p = 3.3 \times 10^{-4}$). Among these 44 immune genes, *CD74*, *CCL2*, and *TNFRSF9* had a fold-change expression of >2 (**Figure 2E**, **Supplementary Table 3**). In addition, we discovered that VPA could increase the MHC I expression level in a dose-dependent manner (**Supplementary Figure 4A**) with 2 mM of VPA exposure inducing a 2.1-fold increase expression of MHC I (relative mean of fluorescence intensity measured by FACS). More importantly, VPA also induced a 2.7-fold increase expression of MHC I in 4T1-derived MammoSpheres (MSs) (**Supplementary Figure 4B**) which harbored a high proportion of CSC-like cells expressing aldehyde dehydrogenase 1 (ALDH1) in permissive culture conditions, in the presence of high doses of TGFβ and TNFα (**Supplementary Figures 5A–D**).

The finding of MHC I expression in "stemness" conditions, led to the hypothesis that VPA treatment could promote directly immune response-associated gene expression, which would improve immune-recognition of CSC-like 4T1 cells by T-cells.

To test this hypothesis, we wished to eliminate the possibility that VPA alone had an effect on tumor growth. We therefore performed tumorigenicity experiments in which we have evaluated the effects of VPA alone on 4T1 tumor size or on metastatic spread to the lungs. In the experiments using VPA alone, there was no significant effect on tumor reduction ($p = 0.73$; **Supplementary Figures 6A,B**), nor on the occurrence of metastatic spread ($p = 0.54$; **Supplementary Figure 6C**) and no effect on $CD4^{+}$ or $CD8^{+}$ T-cells, Tregs or MSCS (**Supplementary Figure 6D**). These data suggested that VPA alone in this dose was insufficient for inducing an immune response against the 4T1 model of breast cancer.

These results prompted us to investigate a combinatory immune strategy using an iPS cell-based cancer vaccine along with the VPA.

Anti-tumor Effects of Murine iPS Cell-Based Vaccines Combined With VPA in an Autologous or Allogeneic Context

To confirm the hypothesis of the occurrence of the immune response along with the anti-tumor effect using the combined

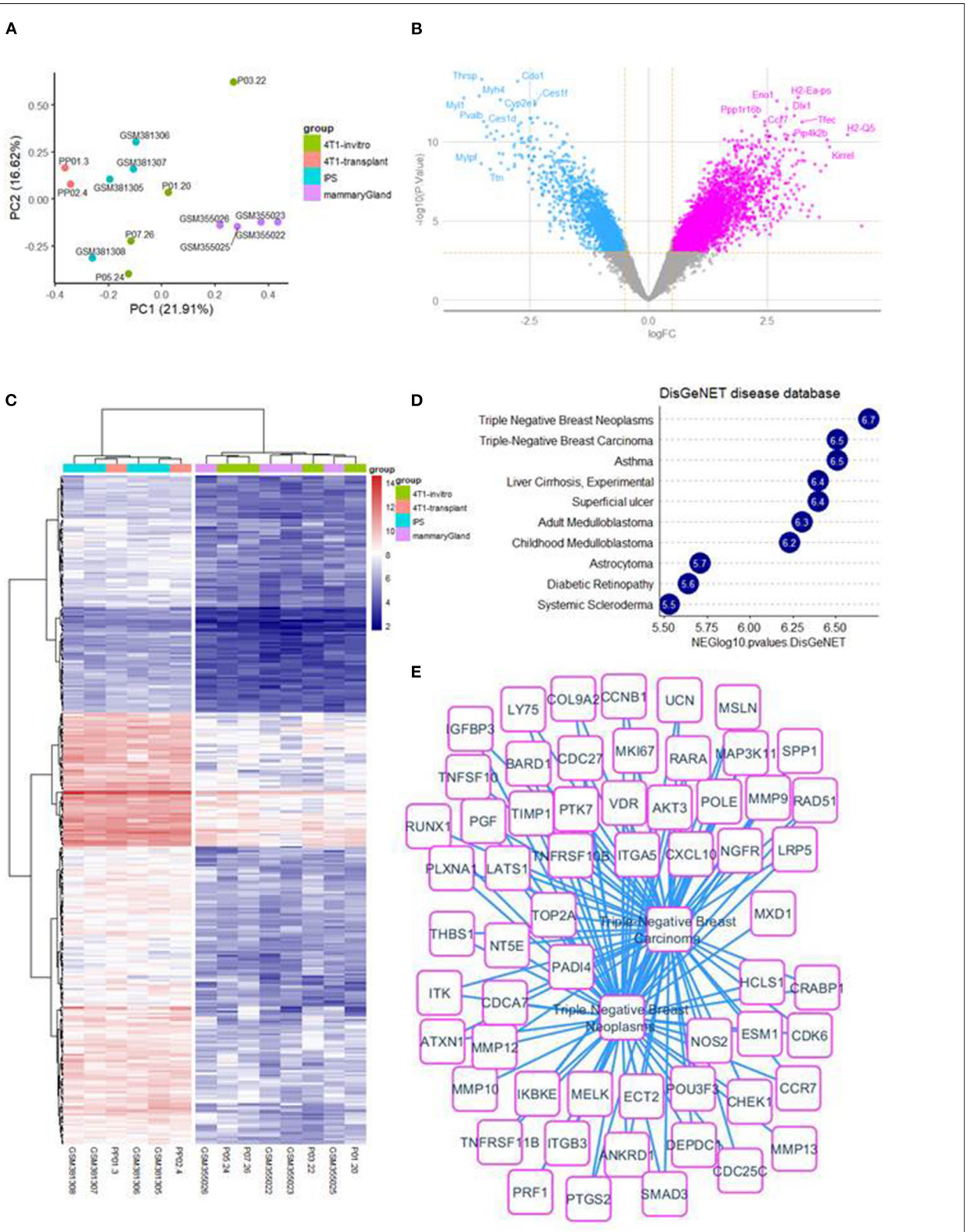

FIGURE 1 | 4T1-transplant samples involved a TNBC up regulated expression profile: **(A)** whole transcriptome principal component analysis stratify on experimental groups. **(B)** Volcanoplot of LIMMA analysis comparing 4T1-transplant and 4T1 *in vitro*. **(C)** Expression heatmap on up regulated genes between 4T1-transplant and 4T1 *in vitro* condition. **(D)** Barplot of functional enrichment performed on DisGeNET with up regulated genes in 4T1-transplant condition. **(E)** Functional enrichment network involving triple negative breast cancer expression profile for 4T1-transplant up regulated genes.

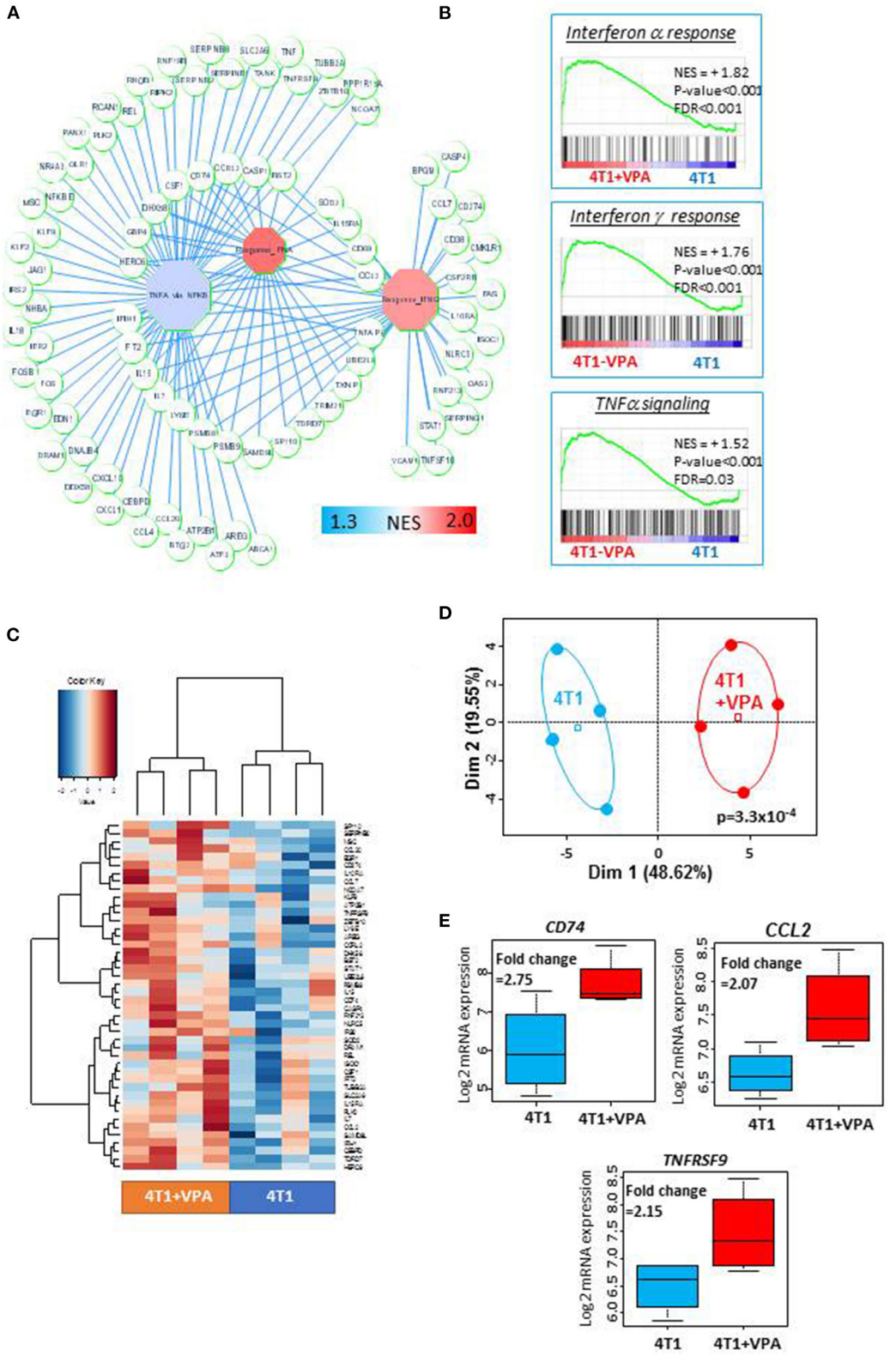

FIGURE 2 | Treatment with valproic acid (VPA) induced an upregulation of the immune response in 4T1 cells *in vitro*: Transcriptome analysis was performed on 4T1 cells treated with or without 0.5 mM of valproic acid for 10 days. Microarray probes were synthetized, labeled (Affymetrix microarray station, Affymetrix, CA) and

(Continued)

FIGURE 2 | hybridized on a Mouse Clariom S microarray (Thermo Fisher Scientific). The CEL files of microarray data obtained from the Affymetrix platform were normalized using the RMA method in Affymetrix Expression Console software (Affymetrix, CA). **(A)** A representation of the immune gene network upregulated by *in vitro* VPA treatment of 4T1 cells. Octagons represent enriched immune modules; circles are genes, which are connected to their respective enriched modules by blue edges (NES, normalized enrichment score). **(B)** Immune gene sets that were significantly enriched in VPA-treated 4T1 cells compared to untreated 4T1 cells (NES, normalized enrichment score, *p*-value and FDR were obtained by a hypergeometric test performed using the MSigDB 6.1 Hallmark database). **(C)** Expression heatmap depicting the immune genes that were significantly overexpressed in VPA-treated 4T1 cells (SAM algorithm, unsupervised classification on Euclidean distances with Ward method). **(D)** Unsupervised principal component analysis performed on upregulated immune genes in VPA-treated 4T1 cells. **(E)** Boxplot of three immune genes that were found to be significantly overexpressed as a result of VPA treatment (fold-change >2).

strategy, we immunized BALB/c immunocompetent mice with the vaccine combination approach, involving 2-weekly 2×10^6 allogenic C57BL/6 derived-miPSCs pre-treated by VPA followed by transplantation of 4T1 cells into the fad pad (**Supplementary Figure 7**). After implantation of the tumor, mice were treated orally by VPA (4 mg/mL) during 15 days. Anti-tumor efficacy of this immune prevention strategy was compared with a preventive therapy using C57BL/6-derived miPSCs without VPA treatment and with VPA treatment alone without vaccination.

Overall these experiments were conducted using 31 BALB/c mice divided into four groups including PBS group ($n = 7$), VPA alone group ($n = 8$), C57BL/6 derived-miPSCs group ($n = 8$) and the group of mice treated with C57BL/6 derived-miPSCs and VPA ($n = 8$). All mice were challenged with 5×10^4 4T1-GFP-Luc cells. At day + 21 after tumor implantation, the group of mice treated with VPA alone and miPSC-based vaccine alone showed 21.7 and 18% reduction, respectively, in their tumor volume which was not significant as compared to those observed in control mice, On the other hand, in mice receiving miPSC with VPA, the tumor volume was significantly smaller with a reduction of 47.6% compared to control mice (104 ± 5.7 vs. 258 ± 12 mm^3, $p < 0.001$; **Figures 3A,B**).

Vaccination experiments were then investigated (i) to explore the effectiveness of the combinatory miPSCs + VPA approach as compared to the regimen using miPSCs alone on tumor size, survival rate and metastases, and (ii) to compare allogeneic C57BL/6-derived miPSCs based vaccine with autologous BALB/c-derived miPSCs.

To this purpose we have used miPSC from BALB/c fibroblasts (**Supplementary Figure 8A**) which exhibited classical pluripotency characteristics (**Supplementary Figures 8B,C**) and generated teratomas *in vivo* (**Supplementary Figure 8D**). Naïve immunocompetent BALB/c mice were pre-immunized every 2 weeks with 2×10^6 allogeneic or autologous miPSCs with or without VPA treatment followed by tumor challenge. Negative control group was injected with PBS without VPA treatment.

As shown in **Figures 3C,D**, the combinatory treatment with miPSCs and VPA allowed a better reduction in the tumor burdens, as compared to control as well as mice vaccinated without VPA treatment. We observed a higher tumor reduction in mice treated with allogeneic miPSC + VPA (61% of reduction; $p < 0.0001$) as compared to mice treated with autologous miPSC + VPA (48% of reduction; $p = 0.0018$).

Experiments evaluating the long-term survival (>50 days) showed that vaccination with miPSC conferred a significant improvement in survival rate over the control group (**Figures 3E,F**). The most significant survival benefit was observed when VPA was combined with the allogenic vaccine ($p = 0.0026$, 100% of survival) (**Figure 3G**). In contrast survival rate was less pronounced with the autologous vaccine combined with VPA ($p = 0.0912$; **Figure 3H**).

We confirmed the efficacy of the combination regimen of allogenic miPSC and VPA in BALB/c mice that were treated using the same protocol as previously by monitoring the tumor volume until day +20, in vaccinated mice (C57BL/6-derived miPSCs + VPA; $n = 8$) and unvaccinated mice ($n = 7$) (**Supplementary Figure 9**). After 3 days, all mice from both groups presented tumors of similar size. Tumors from control mice grew dramatically over the remainder of the experiment. Instead, in mice treated with allogeneic C57BL/6-derived miPSC+VPA, tumors grew much more slowly, and indeed, even shrank in 4 of the 8 treated mice. In one mouse, the tumor completely disappeared, while the other three mice demonstrated a partial regression in tumor size, with final volumes of <35 mm^3 (**Supplementary Figure 9**).

Iterative MiPS-Cell Based Vaccines Promote an Efficient Memory Immune Response to Prevent Tumor Growth and Metastatic Spread

We then asked whether miPS-cell based vaccine could be used in healthy mice treated with iterative injections aiming to induce a durable long-term T-cell memory response. For this purpose, we have chosen to use autologous miPSC to avoid allo-immune response. We inoculated mice with 2×10^6 irradiated BALB/c-derived miPSCs during 6 months, with a total of 6 injections, once every 30 days. Thirty days (**Supplementary Figure 10A**) or 120 days (**Figure 4A**) after the final vaccine inoculation, mice were challenged with 2.5×10^4 4T1Luc cells. All vaccinated mice received 4 mg/mL VPA by oral route starting from the day of tumor injection, and tumor growth was monitored for 26 or 28 days after challenge. All mice primed by miPSCs cells were healthy without any side effects after 6 months of vaccination, which suggest that the protocol proposed was a safe preventive approach.

Indeed, tumor volume was reduced by 64% in mice challenged with 4T1 30 days after the final vaccine dose, compared to unvaccinated mice (695 ± 102 vs. $1{,}968 \pm 96$ mm^3, $p = 0.005$; **Supplementary Figure 10B**). When we increased the time elapsed between the final vaccine dose and tumor implantation to 120 days, we observed again a significant response (46%

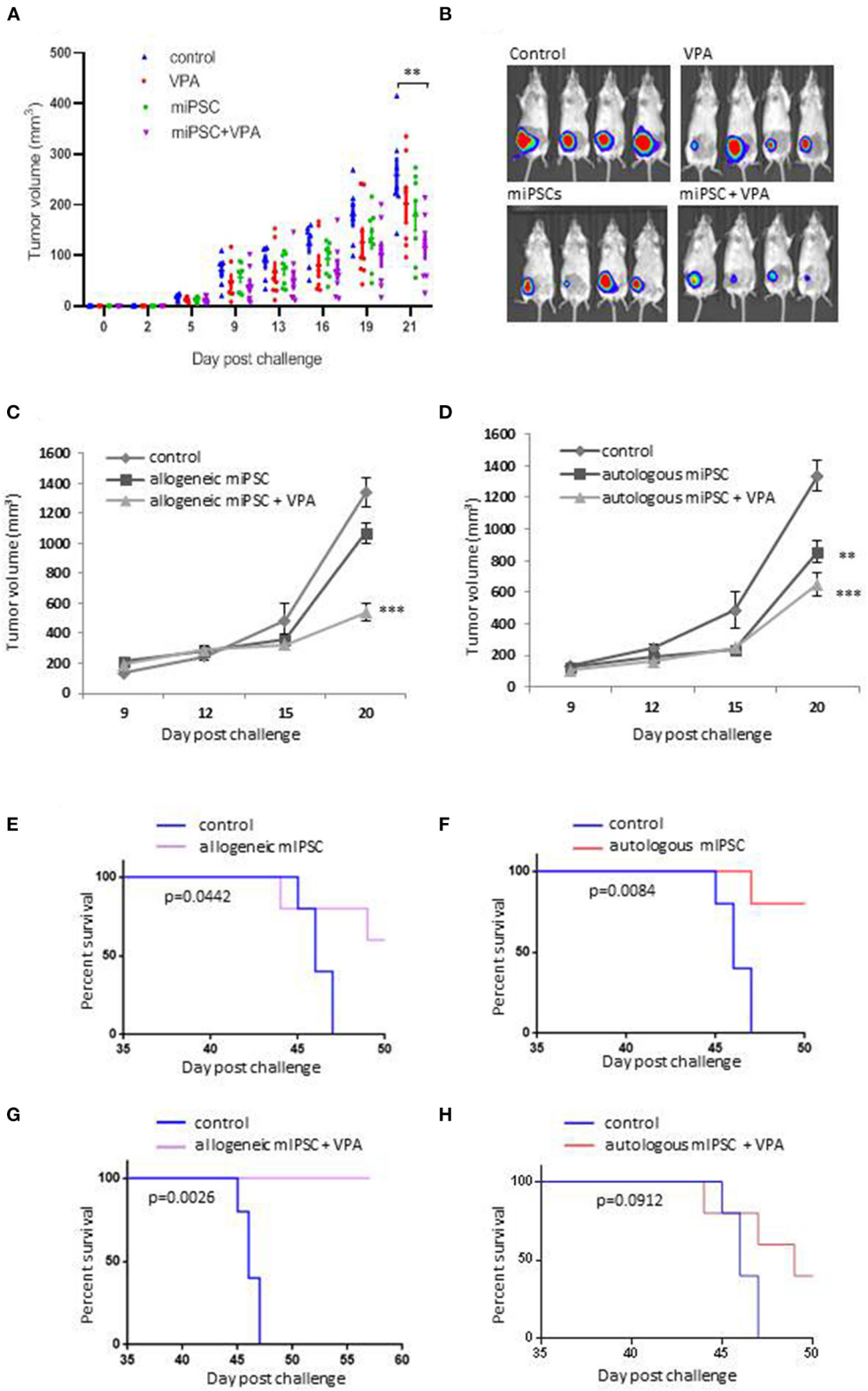

FIGURE 3 | Evaluation of the anti-tumor effects of murine iPSCs derived from BALB/c or C57BL/6 mice. **(A)** Evaluation of the anti-tumor effects of C57BL/6- derived miPSCs murine with or without VPA. Eight to 10 weeks old female BALB/c mice, were divided into 4 groups: group control (PBS; $n = 7$), mice treated with VPA ($n = 8$) or miPSCs alone ($n = 8$), and mice treated with miPSCs + VPA ($n = 8$). Treatment consisted of two sub-cutaneous injections (one-week interval between injections) of

(Continued)

FIGURE 3 | 2×10^6. One week following the second injection, all mice were inoculated with 5×10^4 4T1Luc cells into mammary fat-pad following by 21 days of VPA treatment, orally administered at dose of 4 mg/mL. **(B)** Representative bioluminescence imaging of tumors from mice treated with miPSCs, VPA and miPCs+VPA compared to untreated mice. **(C)** Tumor growth in 8–10 weeks old, BALB/c mice that were immunized with murine C57BL/6-derived iPSCs; with ($n = 5$) or without VPA ($n = 5$), compared to control group ($n = 5$) by using the same protocol as previously. **(D)** Tumor growth in 8–10 weeks old, BALB/c mice that were immunized with murine BALB/c-derived iPSCs; with ($n = 5$) or without VPA ($n = 5$), compared to control group ($n = 5$) by using the same protocol as previously. **(E)** Effect of allogeneic C57BL/6-derived miPSC treatment on the survival of mice BALB/c challenged with 5×10^4 4T1 cells (n=5 per group). **(F)** Effect of autologous BALB/c-derived miPSC treatment on the survival of BALB/c mice challenged with 4T1 cells 5×10^4 4T1 cells (n=5 per group). **(G)** Effect of allogeneic C57BL/6-derived miPSC +VPA treatment on the survival of mice BALB/c challenged with 5×10^4 4T1 cells ($n = 5$ per group). **(H)** Effect of autologous BALB/c-derived miPSC treatment on the survival of BALB/c mice challenged with 4T1 cells 5×10^4 4T1 cells ($n = 5$ per group). ** $p < 0.01$, *** $p < 0.001$.

reduction: 320 ± 23 vs. 600 ± 40 mm^3 for controls, $p < 0.001$; **Figure 4B**).

We performed an in-depth investigation of the tumor dissemination status in mice for which 120 days elapsed between their final vaccine dose and tumor challenge. The analysis of metastatic dissemination at + 28 days, revealed a major reduction in the lung metastases in this group compared to controls (**Figure 4C**), with a significant correlation between tumor burden and metastatic spread (**Figure 4D**).

In both cases, repeated doses of miPS-whole cell vaccines mediated a tumor effective immune response that resulted in a significant inhibition of tumor growth and metastatic spread compared to the control group.

We then analyzed the tumors with regard to their tumor infiltrating lymphocytes (TILs) contents and tumor immune micro-environment (TIME) landscape. Treatment with miPSCs combined with VPA was correlated with a significant increase in the frequency of $CD4^+$ or $CD8^+$ T cells in the tumors (**Figure 4E**) and in the spleens (**Supplementary Figures 11A,B**). Furthermore, we observed a significant decrease in the frequency of $PD1^+$ $CD4^+$ cells in the spleen (**Supplementary Figure 11C**), as well as a decrease in PDL1 expression in tumor cells (**Figure 4F**), suggesting strongly that the vaccination protocol had overcome the T-lymphocyte anergy.

The analysis of the cellular TME components showed that the combined miPSCs and VPA administration was able to convert the immune- repressive TME into an active one by a significant decrease of the CD4+ CD25+FoxP3+ Tregs frequency (**Figure 4G**) as well as the decrease of $Arg1^+$ CD11b+ Gr1+ pre-MDSCs and $Arg1^+$ CD11b+ Ly6+ gram MDSCs frequency (**Figure 4H**) which are the main immunosuppressive actors of the TME.

Repeated doses of autologous miPSC were found to mediate additional long-term effects on $CD22.2^+$ B-lymphocytes. Specifically, we observed a significant increase in $CXCR5^+$ B cells in vaccinated mice as compared to controls (**Figure 4I**), suggesting that B-lymphocytes had migrated to tumor sites as a result of the combined therapy. Level of T-Effector Memory Cells (TEMs) was also evaluated and showed an increase of CD44+ CD62L- CCR7low CD127low TEMs frequency in the tumors (**Figure 4J**). The strong decrease of KLRG1 expression on TEMs into the tumors (**Figure 4K**) and the decrease of PD1 expression by TEMs (CD44+CD62L-CCR7 low CD127 low) from spleens (**Supplementary Figure 11D**) suggested that the immune memory had reverted to an active state, decreasing both senescence and anergy.

These results provide strong evidence that a sequential miPS-whole cell based vaccination led to the establishment of long-term immune memory without any side effects with the activation of an effective anti-tumor immunity against metastatic spread. A strong benefit was also seen in survival data, as our vaccination protocol dramatically improved the median long-term survival of treated mice as compared to controls (54 days in control group vs. not reached in vaccinated group) (**Supplementary Figure 10C**).

Finally, we wished to compare the efficacy of the vaccination protocol against the tumor challenge by 2.5×10^4 MammoSpheres-derived 4T1 cells (**Supplementary Figure 12A**). As we found with our previous results, immunization with miPSCs led to a significant ($p < 0.0001$) and highly effective immune response to MS-derived 4T1 cancer cells. The total tumor burden in treated mice was reduced by 83% compared to controls, with a massive reduction in tumor sizes (**Supplementary Figures 12B,C**). The long-term immune memory generated by our protocol also significantly protected the mice from developing lung metastases (**Supplementary Figures 12D,E**).

To investigate the underlying mechanisms of the long-term immune protection that resulted from the vaccination protocol, we performed a transcriptome analysis of tumors from untreated mice and from mice primed with 6 doses of miPSCs and VPA in order to find genes that were differentially expressed between the two conditions. As can be seen in **Supplementary Table 4** and **Figure 5A**, 206 genes were found to be differentially expressed in a highly significant manner.

Of the genes that were upregulated in treated mice, 98 were implicated in the immune response to cytokines and lymphocyte chemotaxis (**Figure 5B**). When we analyzed genes linked to the immune response, we detected a significant enrichment in monocyte-derived dendritic cells [Normalized Enriched Score (NES) = 1.66, $p < 0.001$], T cells (NES = 1.54, $p < 0.001$), and B cells (NES = 1.33, $p < 0.001$; **Figure 5C**). Likewise, a gene network-based analysis supported the major contribution of B-cells and T-cells by highlighting genes associated with these respective infiltrations (**Figure 5D**). Interestingly, a significant upregulation (8.8-fold; $p = 5.1787803E\text{-}5$) of chemokine (C-X-C motif) ligand 13 was detected in tumors that persisted after vaccination (**Supplementary Table 4**). This result was confirmed by qRT PCR, which also revealed a strong upregulation of CXCL13, CXCL9, and CXCL10 in the tumors of vaccinated mice (**Supplementary Figure 13**).

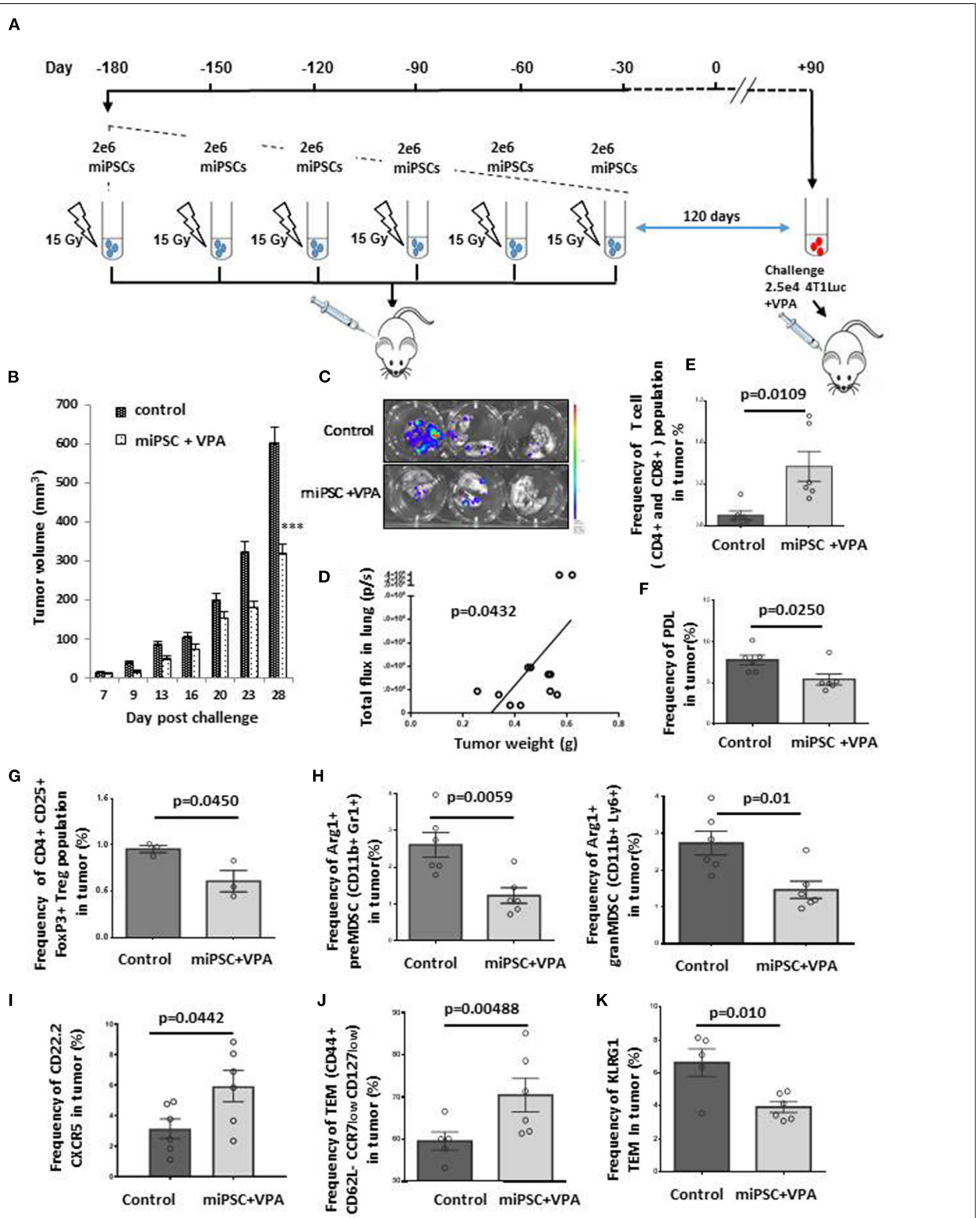

FIGURE 4 | Effective memory immune response following vaccination with autologous miPSCs. Mice were treated ($n = 6$) with sub-cutaneous injections of 2×10^6 irradiated BALB/c-derived miPSCs during 6 months, with a total of 6 injections, once every 30 days. One hundred twenty days after the last vaccine inoculation, mice were challenged with 2.5×10^4 4T1Luc cells and vaccinated mice received 4 mg/mL VPA by oral route starting from the day of tumor injection until the sacrifice. Control mice ($n = 7$) received only PBS. **(A)** Experimental protocol to evaluate *in vivo* immune memory generated by vaccination: BALB/c mice were injected

(Continued)

FIGURE 4 | subcutaneously six times with 2×10^6 miPSCs (15 Gy irradiated) in the right flank. **(B)** At day 28 post-challenge, breast tumors were significantly smaller in mice that had undergone the six-month vaccination protocol compared to unvaccinated mice. **(C)** Bioluminescence images of lungs isolated from miPSC-vaccinated and control mice 28 days after tumor challenge. **(D)** A significant correlation was found between tumor burden and metastatic spread in the lungs of vaccinated mice at day 28 post-challenge. **(E)** The frequency of $CD4^+CD8^+$ cells in tumors of treated and untreated mice, as measured by flow cytometry. **(F)** The frequency of $PDL1^+$ cells in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry. **(G)** The frequency of Treg cells in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry. **(H)** The frequency of $Arg1^+$ preMDSCs and granMDSCs in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry. **(I)** The frequency of $CXCR5^+$ $CD22.2^+$ LB cells in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry. **(J)** The frequency of T-effector memory cells in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry. **(K)** The frequency of $KLRG1^+$ T-effector memory cells in tumors of miPSC-vaccinated mice compared to controls, as measured by flow cytometry, *** $p < 0.001$.

DISCUSSION

In this study, we evaluated for the first time the effects of a combinatory vaccination strategy using autologous and allogeneic iPS-whole cell-based vaccine along with a histone deacetylase inhibitor (HDACi) in cancer development in an aggressive murine breast carcinoma model with metastatic potential. Cancer-stem like cells escape to immune system by different mechanisms in particularly by deregulation of signaling pathways and the silencing of the MHC I expression by several epigenetic perturbations. In order to promote the immune-recognition of cancer cells, our vaccination strategy used the addition of VPA, an HDACi which removes acetyl marks from tagged histones to increase global histone acetylation. Interestingly, HDACi might also work to re-activate gene expression by altering the global nuclear architecture. Thus, increase in histone acetylation can result in a relaxed chromatin configuration, enabling access to transcriptional activators to restore gene transcription. Epigenetic drugs targeting these enzymes can restore, and in some cases induce overexpression of genes that have been epigenetically silenced in both immune and cancer cells (39) including MHC1 molecules (40).

The concept of using iPSCs as a source of tumor-associated antigens (TAAs) (41, 42) with the ultimate goal of eliciting an anti-tumor immune response was previously reported either on the preventive effects of embryonic stem cells on transplantation of cancer cell lines (16–18) or using the autologous anti-tumor vaccines using iPSCs, with TRL9 as adjuvant, in a prophylactic setting in a non-metastatic syngeneic murine cancer cell lines (14, 15). However, none of these existing reports evaluated the anti-metastatic potential of iPS-whole cell vaccines, nor did they determine whether such vaccines had the ability to target aggressive tumors enriched with CSCs-like cells and to modify their microenvironment.

We show in this work for the first time that a combined iPS-cell-based cancer vaccine and HDACi (here valproic acid) strategy was highly efficient to inhibit the growth of an aggressive, poorly differentiated triple-negative breast cancer (TNBC) cell line. This model closely mimics the metastatic human basal-like breast cancer responsible for rapid and lethal metastatic spread *via* a hematogenous route mainly to the lungs (43, 44).

We also uncovered in this work the efficacy of this iPS-cell-based cancer vaccine for its broad mechanism of action by immune modulation effect and its multiple immune stimulatory functions able to modify the tumor microenvironment.

Because the antigen processing ability of CSCs is epigenetically down-regulated, they express low levels of MHC-I molecules, leading to their altered detection by the host immune system (45). This effect is further enhanced by the fact that the surrounding tumor microenvironment is highly immunosuppressive (45). The combination of these two factors is remarkably effective in enabling CSCs to escape the surveillance of an efficient immune system.

We have also explored in this work, the potential of HDACi to increase the efficacy of the miPS-cell-based cancer vaccine. HDACi are known to have multiple biologic effects consequent to alteration in patterns of acetylation of histones, implicating proteins involved in the regulation of gene expression, pathways of apoptosis, cell cycle progression, mitotic division, cell migration, and angiogenesis (46). HDACi were also shown to have potent immunomodulatory activities. There are robust data supporting the use of epigenetic drugs such as HDACi on their ability to modulate immune-cancer cell interactions leading to the reversal of crucial events of immune evasion. Indeed, HDACi were shown to increase the expression of TAAs and specially Cancer Testis Antigens (CTAs) (47) and to increase the expression of perforin in T cells (48). They also increase the antigenic recognition by CTLs (49) by mediating recognition of cancer cells by CTLs (50). It has also been demonstrated that HDACi down regulate MDSC expansion and function, reduce the expression of arginine-1, which are known to impair T cell proliferation and cytokine production (51) and to enhance T-cell chemokine expression (52). HDACi have also been proven to modulate innate host immune cells by increasing the expression of the activating receptor NKG2D on the surface of NK cells (53). It has been previously shown that they enhance NK cell-mediated tumor cell targeting by upregulating the stress-inducing ligands MICA, MICB, and ULBP1-3 in tumor cells permitting a more efficient killing of tumor cells (50). In addition, they have been reported to increase the expression of death-inducing receptors FAS and TRAIL-R2 on cancer cells enhancing the death of cancer cells by NK cells (54).

We observed in our experimental context, that VPA had various mechanisms of action, allowing to improve cancer vaccine efficacy by transforming the TME and to overcome the immunological tolerance of the cancer stemness phenotype by drastically altering the immune system within the TME. Several lines of finding support this assumption. Firstly VPA had the potential to significantly increase the expression of MHC1 in 4T1 cells and in CSC/MS-derived 4T1 cells. It has also increased the expression of MHC2 (CD74), chemokine CCL2, and TNFRSF9,

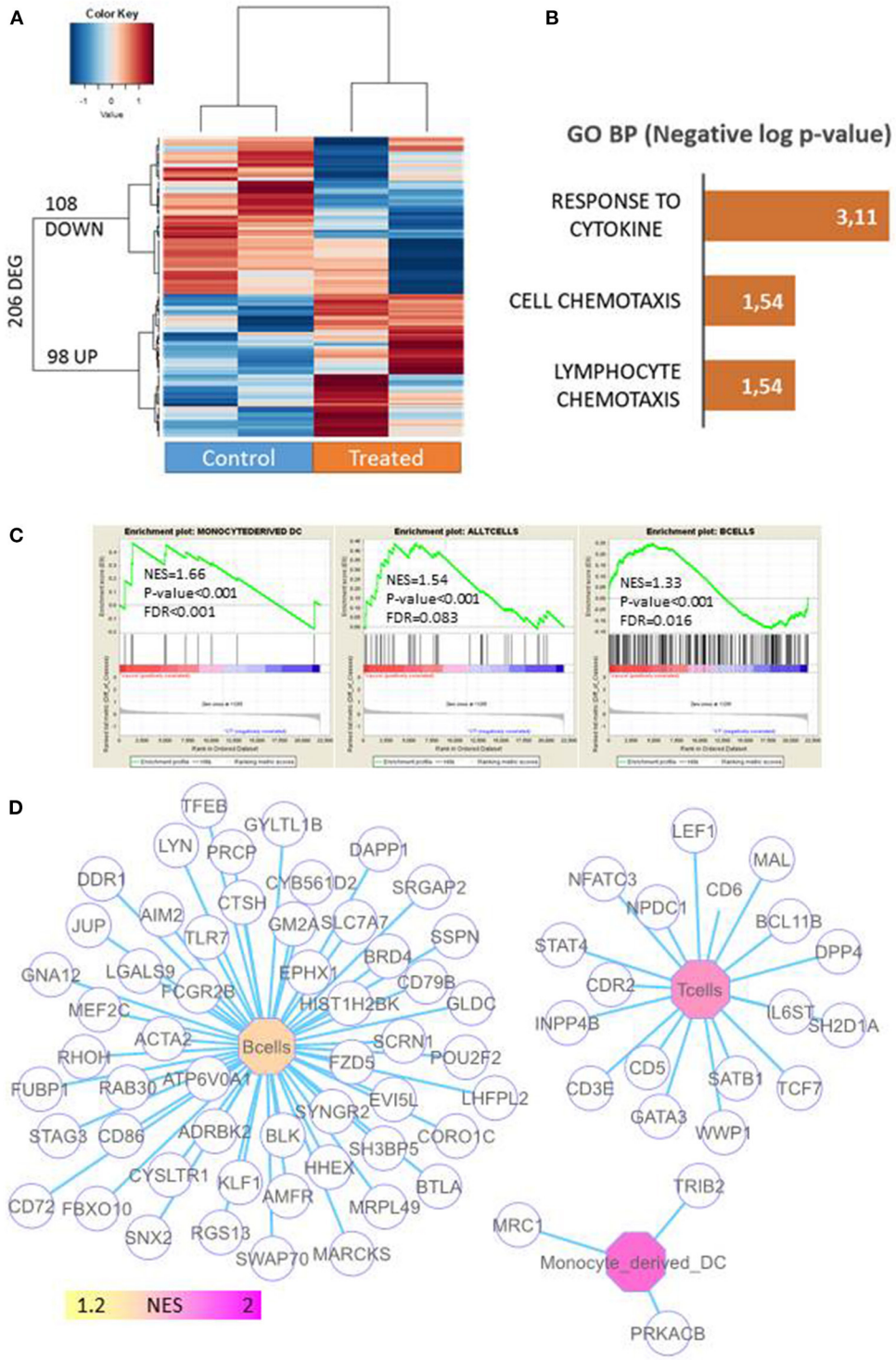

FIGURE 5 | Profiling of immune-associated genes in 4T1 tumors in mice primed with miPSCs. Transcriptome analysis was performed on tumors from untreated mice and from mice primed with 6 doses of miPSCs and VPA in order to find genes that were differentially expressed between the two conditions. Total RNA was extracted from 4T1 tumors in treated and control mice, following the instructions of the manufacturer (TRIzol, Life Technologies). Microarray probes was synthetized by one cycle of RNA amplification in which molecules were labeled in an Affymetrix microarray station (Affymetrix, CA). Labeled microarray probes were hybridized on a Mouse Clariom S (mm10) microarray (Thermo Fisher Scientific). **(A)** Differentially expressed genes (DEGs) between control and vaccinated mice xeno-transplanted with 4T1 cancer cells; expression heatmap was produced with an unsupervised classification algorithm (Euclidean distances, Ward method). **(B)** Barplot of functional enrichment in Gene Ontology Biological Processes of genes that were overexpressed in vaccinated mice. **(C)** Immune profiling carried out by gene-set enrichment analysis on the transcriptome of 4T1-transplanted mice (NES: normalized enriched score). **(D)** Genes associated with the immune network that were enriched in 4T1 tumors from miPSC+VPA-treated mice.

a member of the TNF-receptor superfamily that is known to contribute to the clonal expansion, survival, and development of T cells and to regulate CD28 co-stimulation to promote Th1 cell responses. CCL2 is a chemokine shown to be a potent chemo attractant for several types of immune cells, including NK cells, memory T cells, and dendritic cells within the tumors (55). We also determined according to other observations (40), that VPA promoted the production *in vivo* of multiple chemokines, such as CXCL9, 10, and 13, which enabled significant modifications in the immunosuppressive microenvironment of tumor cells and facilitated the local recruitment of T and B cells within the tumor. VPA was also found to attenuate the immunosuppressive proportion of myeloid-derived suppressor cells (MDSC). This last mode of action is in concordance with previous studies showing that HDACi treatments efficiently decrease Gr-1+ MDSC accumulation in the spleen and tumor bed in BALB/C mice with 4T1 mammary tumors by inducing MDSC apoptosis through mitochondrial Reactive Oxygen Species (ROS) signaling pathway (56).

One important discovery reported here is related to the possibility of generating anti-cancer immunity not just toward "bulk" cancer cells but also toward primitive de-differentiated CSC-like MammoSphere cells. Indeed, in several cancers, gene expression programs have been identified to be similar to those of embryonic pluripotent stem cells, namely, a "stemness" profile which is associated to oncogenic de-differentiation in epithelial cancer progression by a gradual loss of a differentiated phenotype and acquisition of progenitor and stem cell-like features (7, 8, 57).

It is well-established that CSCs are the cause (i) of resistance to "classical" therapies (58–60), (ii) of relapses in several types of aggressive cancers (61–64) and (iii) of metastatic dissemination (65). Tumor cells undergoing EMT are enriched in CSCs with a capacity for early migration and long-term persistence in a dormant stage for long periods of time. Such cancers correlate with aggressive, poorly differentiated tumor histology, invasive tumors, and very adverse outcomes (7, 8, 66).

Prior to our study, there had been no evidence presented as to whether immunotherapy vaccine strategies that use iPSCs as the source of TAAs have the ability to specifically target CSCs and/or the CSC niche in TNBC. The 4T1 breast cancer model is well suited for addressing this question as it is known to hijack some of the normal stem cell pathways to increase cellular plasticity and stemness (44). Our combinatory regimen iPSCs + VPA as adjunct had a substantially enhanced anti-tumor effect compared to the vaccine-only treatment or to the use VPA alone, and caused a significant reduction in lung metastases. These results indicate that iPSCs + VPA have significantly modified the immunosuppressive microenvironment within the primary tumor and reduced the number of cancer cells with a stemness/CSC phenotype that were able to migrate to the secondary organs.

In this work, we evaluated two sources of fibroblast-derived iPSCs generated from the BALB/c and C57BL/6 strains of mice, respectively; this enabled us to compare anti-tumor immunity generated in BALB/c mice under autologous vs. C57BL/6 allogeneic conditions. Regardless of the strain of origin, vaccinated mice had significantly smaller tumors compared to unvaccinated controls, and the inclusion of VPA treatment increased the anti-tumoral response considerably. We also found a significant improvement in the survival rate due to vaccination, and this was more pronounced in mice that had been primed with allogeneic material combined with VPA. Allogeneic iPSCs trigger a stronger cellular and humoral alloimmune response due to allo-immunity stimulated by MHC mismatches.

The use of allogenic iPSCs represents a considerable advantage in the development of a scalable cancer stem cell-based vaccine for all solid tumors with stemness feature. Allogeneic iPSCs allow the development of "off-the-shelf" whole cell-based vaccine for curative approaches combined with other therapies. In our experiment design, this allogeneic iPSC-based therapy could be used as an adjuvant approach, in order to prevent the short and long-term risk of relapse and metastasis. Our allogeneic iPSCs-whole cell-based vaccine with VPA could be used at distance of any chemotherapy, in minimal residual disease or in patients with apparent remission to eradicate residual CSCs in multiple cancer sharing stemness feature.

The length of time needed to produce bona fide iPSCs (several months, corresponding to several passages in culture and quality controls to ensure their purity and safety) would render autologous iPSCs highly impractical for curative treatment. However, in patients harboring germline mutations with a high risk of cancer, autologous-derived iPSCs would likely be useful in prophylactic settings. We confirmed the safety of iterative repeated doses of autologous miPSC injection in healthy immune-competent mice, which allowed the generation of a pool of effective memory T cells and B cells against 4T1 breast carcinoma development. Indeed mice that were challenged with 4T1 cells 30 or 120 days after the end of a six-dose vaccination series were able to reject 4T1 cells. These anti-tumoral responses was correlated with a significant increase in the frequency of $CD4^+$ and $CD8^+$ T cells within the tumors and a significant decrease in the tumors of CD4+ CD25+FoxP3+ Tregs, of $Arg1^+$ CD11b+ Gr1+ preMDSCs and of $Arg1^+$ CD11b+ Ly6+ gramMDSCs indicating a negative impact of the treatment on the main immunosuppressive actors of the immune system. We also observed a significant increase in $CXCR5^+$ B cells in vaccinated mice compared to controls suggesting that B-lymphocytes had migrated to tumor sites as a result of our treatment. We also have observed a significant increase in the frequency of CD44+ CD62L- CCR7low CD127low TEMs as well as in KLRG1 expression by TEMs in tumors suggesting that the immune memory had reverted to an active state, decreasing both anergy and senescence.

In our study, we did not observe any significant autoimmunity: immunized mice were generally healthy and presented no clinical evidence of autoimmune diseases. The animals' weight, hair, and musculature were normal. However, more follow-up is needed before iPS whole cell-based cancer vaccines move into clinical testing.

Taken together, our data show the feasibility of creating anti-cancer stem cell immunity through an approach that combines an HDACi and iPSC whole cell-based vaccine. These data

strongly confirm that iPS cell-based vaccine + VPA in immune-competent mice have a broad mechanism action for metastatic aggressive tumors with stemness features. The principal mode of actions are driven by (i) enhanced trafficking with mobilized TILs in response to chemokines, (ii) improved cancer stem cell immune-recognition, (iii) decreased of MDSCs, (iv) recruited B cells into the tumor.

This technique can be easily applied to patients and, because it targets multiple TAAs from CSC-like cells and not from adult normal stem cells, this safe approach significantly decreases the occurrence of metastasis. This combinatory regimen should improve the overall survival of patients with stemness/ CSC-phenotype tumors associated with a dismal prognosis, particularly given that for solid tumors, invasion and metastasis account for more than 90% of mortality (65, 67). This new approach can be an alternative to the use of chemical compounds able to inhibit signaling pathways present in CSCs such as Stat3, mTor, Smo, Notch of Hedgehog inhibitors which are currently under study. Most of them have shown to be toxic and to cause collateral damages since all these signaling pathways are also heterogeneously expressed in the adult stem cell populations (68).

These beneficial properties of our approach make this combinatory regimen a potentially powerful option for a new concept of active immunotherapy that could be deployed shortly after conventional primary treatment of cancer or in combination with conventional therapies or "checkpoint inhibitors," which are currently under intensive investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the Val de Marne.

AUTHOR CONTRIBUTIONS

FG, AB-G, and AT: direction and financing of the project. JA and MK: generation, production, and characterization of miPSCs. AA, MK, and FG: mouse experiments. MK and AA: molecular and cellular experiments and flow cytometry. CD: microarray and bioinformatic analysis. FG, AB-G, AT, MK, and AA: writing of the article.

FUNDING

This work was performed with grants from ANR Programme d'Investissements d'Avenir of the INGESTEM National Infrastructure Program (ANR-11-INBS-0009-INGESTEM), Inserm, University Paris Saclay.

ACKNOWLEDGMENTS

We thank Benoit Peuteman for animal care; Paule Opolon for anatomopathological analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.729018/full#supplementary-material

Supplementary Figure 1 | Development and ECM functional network up regulated in 4T1-transplant: **(A)** Barplot of functional enrichment performed on GOBP database (development and morphogenesis components). **(B)** Functional enrichment network implicated in developmental processes. **(C)** Barplot of functional enrichment performed on MSigDB database. **(D)** Functional enrichment network comprising relations with the microenvironment.

Supplementary Figure 2 | Cell mobility and cell migration functional enriched network up regulated in 4T1-transplant. **(A)** Barplot of functional network performed on GOBP database. **(B)** Functional enrichment network implicated in cell migration and cell mobility.

Supplementary Figure 3 | Quantification of CD44/CD24 markers in 4T1 cells by flow cytometry *in vitro* and *in vivo* 12 and 28 days after implantation into the fad pat of BALB/c mice.

Supplementary Figure 4 | VPA treatment increased MHC I expression. 4T1 cells were incubated for 48 h with a dose of 0.2 and 2 mM VPA and MHC1 expression quantified by Flow cytometry using MHC Class I (H-2Kd/H-2Dd), eFluor 450 antibody. 4T1 MammoSpheres (MSs) were produced during 9 days in low-attachment 6-well plates at density of 100,000 cells per well in MEF-conditioned medium (3/4 MEF-conditioned medium + 1/4 mES medium + 4 ng/mL bFGF), and addition of TNF-alpha (20 ng/mL), and TGF-β 1 (10 ng/mL). **(A)** Increase in MHC I expression on the surface of 4T1 cells as a result of treatment with different doses of VPA (0, 0.2, and 2 mM), as revealed by flow cytometry. **(B)** Effect of treatment with 2 mM VPA on the expression of MHC I in adherent 4T1 cells and 4T1-derived MSs.

Supplementary Figure 5 | Generation of MammoSpheres from the 4T1 cell line. 4T1 MammoSpheres (MSs) were produced in 3D culture condition using low-attachment 6-well-plates. 4T1 cells were cultured for 9 days at a density of 100,000 cells per well in MEF-conditioned medium (3/4 MEF-conditioned medium + 1/4 mES medium + 4 ng/mL bFGF), and addition of TNF-alpha (20 ng/mL), and TGF-β 1 (10 ng/mL). **(A)** Images of the morphology of adherent 4T1 cells and 4T1-derived MSs in 3D culture conditions, cultured with or without TGFβ + TNFα (magnification ×20). **(B)** Mammosphere-formation efficiency (calculated by Cell Selector Software) indicates the number of mammospheres of different sizes obtained with TGFβ + TNFα treatment. **(C)** Quantification of ALDH1 activity by flow cytometry in adherent 4T1 cells and in 4T1-derived mammospheres cultured with or without TGFβ + TNFα. **(D)** Percentage of $ALDH1^+$ cells among adherent 4T1 cells and 4T1-derived mammospheres cultured with or without TGFβ + TNFα. Results are shown from three independent experiments.

Supplementary Figure 6 | VPA-only treatment did not hinder *in vivo* tumor growth. Sixteen 8–10 weeks old females BALB/c mice, were divided into 2 groups: group control and mice treated with VPA orally administered at dose of 4 mg/m. All mice were inoculated with 5×10^4 4T1Luc cells into mammary fat-pad and tumors monitored for 21 days. **(A)** Tumor growth (mm^3) in mice treated with VPA compared to control mice. **(B)** IVIS imaging of VPA-treated and untreated mice at day 21. **(C)** Lung metastases in VPA-treated and untreated mice were quantified using bioluminescence imaging. Regions of interest (ROI) for pulmonary metastases in these two groups were calculated by Living Image Software. **(D)** Effects of VPA treatment on the frequencies of $CD4^+$ cells, $CD8^+$ cells, Tregs, and MDSCs compared to controls, as quantified by flow cytometry.

Supplementary Figure 7 | Vaccination and challenge protocol. Murine iPSCs were incubated for 24 h with 0.5 mM of VPA and irradiated at the dose of 15 Gy. Irradiated iPSCs were injected as a vaccine into BALB/c mice twice, with a one-week interval between doses. Mice were then challenged with 5×10^4 4T1

cells 1 week after the final dose. VPA was orally administered to vaccinated mice through their drinking water at a dose of 4 mg/mL.

Supplementary Figure 8 | Characterization of murine induced pluripotent stem cells derived from BALB/c fibroblasts. Primary fibroblasts from BALB/c mice were reprogrammed into pluripotency by ectopic expression of OCT4, SOX2, c-MYC, and KLF4 using a Cre-Excisable Constitutive Polycistronic Lentivirus (EF1alpha-STEMCCA-LoxP backbone from Millipore). **(A)** Morphological view under the microscope of miPSCs expanded on mouse embryonic fibroblasts. **(B)** Expression of the key pluripotency markers NANOG and OCT4 as revealed by immunofluorescence; DAPI was used as counterstain. **(C)** Expression of SSEA1 in cell membranes as quantified by flow cytometry. **(D)** Teratoma formation assays, showing differentiation into ectodermal, endodermal, and mesodermal tissues.

Supplementary Figure 9 | Tumor volumes of BALB/c mice treated with allogeneic C57BL/6-derived miPSCs + VPA compared to those of untreated mice. Eight to ten week old, females BALB/c mice were divided into 2 groups: group control (PBS; $n = 7$), and mice treated with miPSCs + VPA ($n = 8$). Treatment consisted of two sub-cutaneous injections (1-week interval between injections) of 2×10^6 miPSCs. One week following the second injection, all mice were inoculated with 5×10^4 4T1Luc cells into mammary fat-pad following by 20 days of VPA treatment, orally administered at dose of 4 mg/m. The data represent the mean ± SEM of tumor volumes.

Supplementary Figure 10 | Effective memory immune response following vaccination with miPSCs. Mice from treated group ($n = 4$) received 2×10^6 irradiated BALB/c-derived miPSCs during 6 months, with a total of 6 injections, once every 30 days. Thirty days after the last vaccine inoculation, mice were challenged with 2.5×10^4 4T1Luc cells and vaccinated mice received 4 mg/mL VPA by oral route starting from the day of tumor injection until the sacrifice. Control mice ($n = 4$) received only PBS. **(A)** Experimental vaccination protocol evaluating induced *in vivo* cell memory immune response: BALB/c mice were injected subcutaneously six times with 2×10^6 miPSCs (15 Gy irradiated) in the right flank. **(B)** At day 26 post-challenge, breast tumors were significantly smaller in mice that had undergone the 6-month vaccination protocol compared to unvaccinated mice ($n = 5$ per group). **(C)** Survival rate of mice treated by miPSC + VPA or by PBS (control group).

Supplementary Figure 11 | Immune cell profiling of the spleens of mice treated with miPSCs + VPA **(A,B)** Frequency of $CD4^+$ and $CD8^+$ T cells in the spleens of mice treated with miPSCs+VPA compared with the control group, as measured by flow cytometry. **(C)** Frequency of PD1 expressed on cell membranes of $CD4^+$ T cells, as measured by flow cytometry. **(D)** Frequency of $PD1^+$ T-effector memory ($CD44^+CD62L^-CCR7^{low}CD127^{low}$) cells in the spleen, as measured by flow cytometry.

Supplementary Figure 12 | Effective memory immune response following vaccination with miPSCs; Mice ($n = 6$) were treated with 2×10^6 irradiated BALB/c-derived miPSCs during 6 months, with a total of 6 injections, once every 30 days. One hundred twenty days after the last vaccine inoculation, mice were challenged with 2.5×10^4 4T1Luc cells cultured in mammo sphere condition. Mammo sphere were performed from 4T1 that cultured for 9 days at density of 100,000 cells per well in MEF-conditioned medium (3/4 MEF-conditioned medium + 1/4 mES medium + 4 ng/mL bFGF), and addition of TNF-alpha (20 ng/mL), and TGF-β 1 (10 ng/mL). Mammo spheres were dissociated in PBS/EDTA before injection into both mammary fat-pad glands. Vaccinated mice received 4 mg/mL. VPA by oral route that was started from the day of tumor injection until the sacrifice. Control mice ($n = 6$) received only PBS. **(A)** Experimental protocol to evaluate *in vivo* immune memory generated by vaccination: BALB/c mice were injected subcutaneously six times with 2×10^6 miPSCs (15 Gy irradiated) in the right flank. **(B)** At day 28 post-challenge, breast tumors were significantly smaller in mice that had undergone the 6-month vaccination protocol compared to unvaccinated mice. **(G)** Bioluminescence imaging of tumors from mice treated with miPSCs + VPA compared to untreated controls. **(H)** Bioluminescence imaging of lungs isolated from vaccinated and control mice 28 days after challenge. **(I)** A significant correlation was found between tumor burden and metastatic spread at day 28.

Supplementary Figure 13 | CXCL9, CXCL10, and CXCL13 chemokines mRNA expressions in tumors from miPSC + VPA treated ($n = 6$) and control ($n = 6$) mice. Mice ($n = 6$) were treated with sub-cutaneous injections of 2×10^6 irradiated BALB/c-derived miPSCs during 6 months, with a total of 6 injections, once every 30 days. One hundred twenty days after the last vaccine inoculation, mice were challenged with 2.5×10^4 4T1Luc cells and vaccinated mice received 4 mg/mL VPA by oral route starting from the day of tumor injection until the sacrifice. Control mice ($n = 6$) received only PBS. After 28 days RNAs of tumors were extracted for the quantification of CXCL9, CXCL10, and CXCL13 mRNA by real time RT-PCR.

Supplementary Table 1 | List of antibodies; Names, references, and the dilution used.

Supplementary Table 2 | Genes found up regulated in 4T1-transplant samples as compared to 4T1-*in vitro* done by LIMMA analysis: for each up regulated genes columns present respective statistics obtained by LIMMA algorithm: $\log_2$ Fold Change, average expression in microarray, *t*-statistics, *p*-value, False discovery rate adjusted *p*-values and *B*-statistics.

Supplementary Table 3 | Differentially expressed immune genes that were upregulated in 4T1 cells by treatment with valproic acid. The list highlights immune-associated genes that were found to be significantly overexpressed in the treatment group using the Significance Analysis of Microarray algorithm with a false discovery rate of <5%. Columns include: gene symbol, gene description, gene ID number, and the fold-change in expression between the VPA-treated group and untreated controls.

Supplementary Table 4 | Genes that were differentially expressed between vaccinated and control mice that developed 4T1-derived tumors. Differentially expressed genes are listed with their gene symbol, description, the fold-change in expression found in transcriptome experiments (Vaccine/Control), and the *p*-value of the comparison.

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frontiers in Medicine

MINI REVIEW
published: 18 January 2022
doi: 10.3389/fmed.2021.774618

In vivo Gene Therapy to the Liver and Nervous System: Promises and Challenges

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In vivo genetic engineering has recently shown remarkable potential as a novel effective treatment for an ever-growing number of diseases, as also witnessed by the recent marketing authorization of several *in vivo* gene therapy products. *In vivo* genetic engineering comprises both viral vector-mediated gene transfer and the more recently developed genome/epigenome editing strategies, as long as they are directly administered to patients. Here we first review the most advanced *in vivo* gene therapies that are commercially available or in clinical development. We then highlight the major challenges to be overcome to fully and broadly exploit *in vivo* gene therapies as novel medicines, discussing some of the approaches that are being taken to address them, with a focus on the nervous system and liver taken as paradigmatic examples.

Keywords: gene therapy, liver, central nervous system, gene editing, translational medicine

OPEN ACCESS

Edited by:
Nuno M. Neves,
University of Minho, Portugal

Reviewed by:
Haihui Pan,
George Washington University, United States
Hai Bac Tran,
University of Adelaide, Australia

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *12 September 2021*
Accepted: *16 December 2021*
Published: *18 January 2022*

Citation:
Cantore A, Fraldi A, Meneghini V and Gritti A (2022) In vivo Gene Therapy to the Liver and Nervous System: Promises and Challenges. Front. Med. 8:774618. doi: 10.3389/fmed.2021.774618

INTRODUCTION

Gene therapy (GT) has recently gained renewed interest and shown remarkable potential as a novel effective treatment for an ever-growing number of diseases, as also witnessed by the recent marketing authorization of several gene therapy products (1). *In vivo* genetic engineering, i.e., GT, involves the direct delivery of a GT medicinal product (GTMP) to patients either *in situ* in anatomically defined locations or systemically to reach organs or tissues such as central and peripheral nervous system (CNS, PNS), liver, muscles, and lungs. Emerging technologies for targeted gene editing are complementing the scope of conventional gene transfer, opening the way to precise gene correction that allows to silence, activate, or rewrite loci of interests in the genome. The GTMP may comprise a virus-derived or non-viral vehicle bearing a transgene expression cassette (gene transfer) or engineered site-specific nucleases or genetic/epigenetic modifiers with or without an exogenous DNA to be introduced into the host cells' genome (gene editing) (2–4). Short interfering RNAs (siRNAs) will not be considered here as GTMPs.

In vivo genetic engineering aims at genetically modifying somatic cells to: (i) treat genetic diseases, by adding functional genes (gene addition) or replacing dysfunctional ones (gene replacement), correcting or disrupting mutated disease-causing genes (gene subtraction) through pre-natal, post-natal or adult intervention; (ii) promote endogenous regeneration by delivering factors for tissue protection/engineering; (iii) tackle cancer by direct/indirect tumor cell elimination, including the use of oncolytic vectors (this will not be discussed here).

The most widely used delivery system for *in vivo* GT among viral vectors are adeno-associated viral (AAV) vectors (5). Lentiviral vectors (LV) are so far mostly used for *ex vivo* GT approaches, i.e., genetic engineering of cells *in vitro* and infusion of the modified cells back to patients, with only few examples related to *in vivo* delivery at the pre-clinical or early clinical stage (6, 7). Lipid nanoparticles (LNP) or chemical conjugates are used for small RNA delivery (8). Non-viral mediated delivery of genome editing components is generally at an earlier stage of development. The vast majority of current clinical trials rely on gene addition, only a few of them are based on gene editing strategies.

The availability of programmable nucleases, such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and, more recently, clustered regularly interspaced short palindromic repeat (CRISPR)–Cas-associated nucleases, has greatly expedited the progress of gene editing from concept to clinical practice (4, 9). Engineering of the Cas9 bacterial adaptive immunity response against phages allowed for the development of methods to generate sequence-specific modifications based on a single-guide RNA complementary to the target genomic sequence. In the last decade, CRISPR/Cas9 systems have been applied to genome and epigenome editing in order to disrupt genes, correct mutations, and silence disease-associated factors in different genetic and sporadic conditions. Genome editing has been predominantly performed *ex vivo*, however a few examples of *in vivo* gene editing exist in early-stage clinical trials.

Here we highlight the major hurdles currently limiting the full potential of *in vivo* genetic engineering (**Figure 1**) and review some possible solutions, with a focus on CNS and liver taken as paradigmatic examples.

COMMERCIAL AND CLINICAL STAGE PRODUCTS

In vivo GT to the Nervous System

Currently, there are 3 commercial *in vivo* GT products and many more in clinical development (**Table 1**) (10). AAV vector-mediated gene replacement of a functional enzyme of the retinal pigment epithelium, or the regulatory protein survival of motor neuron is at the bases of Luxturna and Zolgensma, indicated for an inherited form of retinal blindness (Leber congenital amaurosis, LCA) or the genetic neurodegenerative disease spinal muscular atrophy, respectively (11, 12). Luxturna is administered *in situ* in the subretinal space, while Zolgensma is delivered systemically. In both cases, long-lasting therapeutic benefit has been shown, with remarkable recovery of vision and motor functions, respectively. Imlygic is an oncolytic vector indicated for melanoma (13).

Encouraging results have also been shown for Duchenne muscular dystrophy by systemic delivery of AAV vectors expressing short forms of dystrophin in early-stage clinical trials (14). Systemic, intrathecal, and intraparenchymal administration of AAV vectors is under early clinical testing for several neurodegenerative diseases, both genetic early-onset (mucopolysaccharidoses (MPS), globoid leukodystrophy (GLD), Fabry disease, Canavan disease) and non-genetic adult-onset diseases [e.g., Parkinson disease (PD), Alzheimer Disease (AD)] (15). Clinical trials involving LV as delivery systems for *in vivo* GT are currently limited to PD, which benefits from intrastriatal injections of a LV coding for three genes essential for dopamine synthesis (16).

EDIT-101 is a gene-editing drug to treat LCA10 with Centrosomal Protein 290 (CEP290)-Related Retinal Degeneration (17). The approach is based on AAV-mediated single-dose subretinal delivery of a CRISPR-Cas9 system designed to excise the intronic IVS26 mutation in the photoreceptor CEP290 gene that causes abnormal splicing and termination of translation due to introduced cryptic exon. EDIT-101 recently entered clinical testing and enrolled 18 people with LCA10 (NCT03872479). To date, no study report has been published.

In vivo GT to the Liver

Systemic administration of AAV vectors expressing coagulation factor VIII or IX transgene in hepatocytes is in advanced phase of clinical testing as a treatment for the inherited coagulation disorder hemophilia and showed multi-year reconstitution of therapeutic amounts of the clotting factors, even though a decreasing trend in factor VIII activity has been reported (18–20). A similar strategy is under evaluation for some inherited liver metabolic diseases (such as familial hypercholesterolemia, hyperbilirubinemia, glycogen storage disease type-Ia, ornithine transcarbamilase deficiency) in earlier phase clinical trials (21).

NTLA-2001 is an *in vivo* gene-editing therapeutic that is designed to treat transtyrhetin (TTR)-related hereditary amyloidosis. Systemic administration of LNP delivering CRISPR/Cas9 RNA to the liver resulted in efficient disruption of TTR gene and subsequent reduction of the toxic misfolded TTR amyloid in 6 affected patients (22).

Despite these successes, several challenges remain to be addressed related to efficacy, safety and immunogenicity of *in vivo* GTMP, as well as manufacturing, regulatory aspects and sustainability, the latter not being the focus of this Mini Review. Below, we highlight the major challenges and elaborate on possible solutions to address some of them.

Abbreviations: AAV, adeno-associated viral; AD, Alzheimer Disease; BBB, blood-brain barrier; CNS, central nervous system; CRISPR, clustered regularly interspaced short palindromic repeat; GAA, acid α-glucosidase a pag. 6; GLD, globoid leukodystrophy; GOF, gain-of-function; GT, gene therapy; GTMP, gene therapy medicinal product; i.v., intravenous; LCA, Leber congenital amaurosis; LNP, lipid nanoparticles; LPL, lipoprotein lipase; LPLD, LPL deficiency; LSD, lysosomal storage diseases; LV, lentiviral vectors; MPS, mucopolysaccharidoses; NHP, non-human primate; PD, Parkinson's disease; PNS, peripheral nervous system; TALEN, transcription activator-like effector nucleases; TTR, transthyretin; ZFN, zinc-finger nucleases.

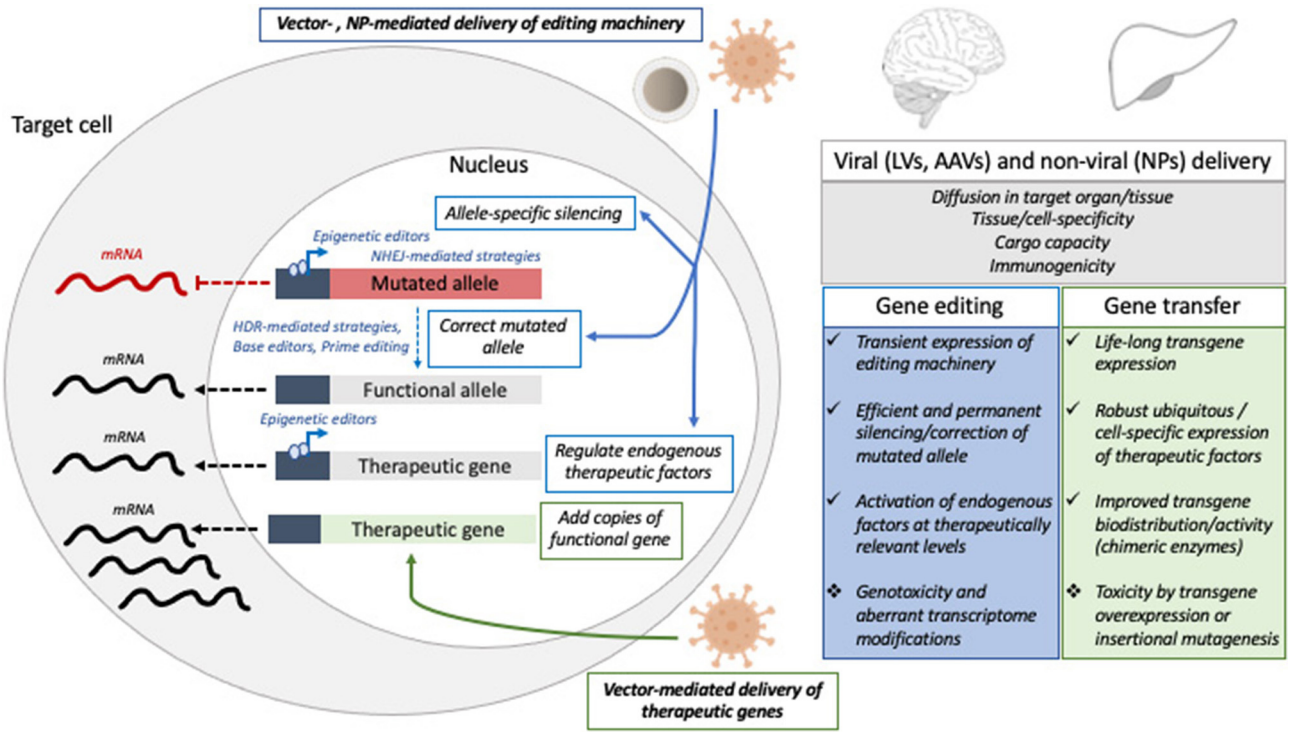

FIGURE 1 | Schematic representation of gene editing and gene transfer approaches tested in pre-clinical and clinical settings to treat liver or CNS disorders, with a list of the major hurdles and challenges that might be addressed to improve the efficacy and safety of *in vivo* GTMP. NP, nanoparticles; LV, lentiviral vectors; AAV, adeno-associated viral vectors; NHEJ, non-homologous end joining; HDR, homology-directed repair.

TABLE 1 | Commercial *in vivo* gene therapy products.

Name	Indication	Vehicle	Mechanism
Luxturna	Leber congenital amaurosis	AAV vector	Gene replacement
Zolgensma	Spinal muscular atrophy	AAV vector	Gene replacement
Imlygic	Melanoma	Herpes simplex type 1 virus-derived vector	Oncolytic vector Gene addition

GENERAL CHALLENGES RELATED TO EFFICACY AND SAFETY OF *IN VIVO* GENETIC ENGINEERING

The efficiency of the genetic engineering (viral gene transfer or genome editing), i.e., the actual quantity of genetically modified cells/genomic loci, may be limiting the efficacy of the procedure depending on the desired therapeutic effect. Tissue/cell-type specificity may be desirable or necessary according to different applications, yet hard to achieve. While the tropism of viral vectors can be controlled to a certain extent, it is currently more difficult to obtain specific targeting by non-viral delivery systems (23, 24). On the other hand, cargo capacity may be more limited for viral than non-viral mediated approaches. Despite a plethora of engineered transcriptional and post-transcriptional control elements available, the strength, cell-type specificity, physiological regulation, duration of transgene expression may all be difficult to control and switching expression on and off at will is yet to be achieved in the clinics (25, 26). For genome editing, efficient but transient expression of the editing machinery should be achieved. For non-monogenic diseases, the target genes to manipulate need to be defined. Ensuring the multi-year, ideally life-long durability of the therapeutic genetic modification is crucial in the context of genetic diseases and needs to rely on transgene integration or stability of the genomic edit in proliferating cells and/or in long-lived target cells; alternatively, safe re-administration of the GTMP has to be ensured (27–29).

Concerning the safety of the *in vivo* genetic engineering, the following risks need to be taken into consideration, carefully evaluated, and reduced to the minimum possible during the research and development phases: the acute responses to the delivery vehicles (30, 31), toxicities due to expression/overexpression of the transgene or other components of the GTMP, possible long-term adverse effects due to genomic insertions of vectors or other components of the GTMP (32),

genotoxicity associated with off-target events, large deletion at the on-target loci, chromosomal rearrangements, and aberrant modifications of the transcriptome (33–36). Moreover, the effects of the GTMP on target cells' biology and functionality should be properly determined. Finally, the innate and adaptive immune responses to the delivery vehicle(s), the transgene product(s), including editing machineries of bacterial origin, and other components of the GTMP need to be assessed to avoid detrimental impacts on both the efficacy and safety of the procedure (37, 38).

MODIFYING THE TRANSGENE TO IMPROVE THE THERAPEUTIC POTENTIAL OF *IN VIVO* GT

One of the main challenges in the clinical translation of *in vivo* GT is the difficulty in achieving and maintaining therapeutic amounts of the corrective gene in targeted tissues, avoiding the use of high dosage and/or repeated administration of the gene delivery vehicle (that, in most cases, is virus-derived), which is not only potentially toxic but also costly. Intra-vascular administration of GTMPs has been extensively tested in preclinical studies and is being exploited in clinical trials to treat the CNS as alternative approach to direct administration (*via* either intraparenchymal or intra cerebrospinal fluid injections), which in principle require lower amount of GMTPs but may represent an invasive approach. However, intra-vascular administration of GTMPs showed limited or no effectiveness on CNS pathology due to the impermeability of the blood-brain barrier (BBB) to large molecules (39). Therefore, this delivery route may require high doses of GTMPs, which may strongly reduce its clinical suitability. A possible strategy to overcome all these limitations is enhancing the therapeutic potential of the GTMP by modifying the expression cassette. Here, we give some examples on how this strategy can be applied to the treatment of inherited diseases due to enzymatic deficiency.

A way to modify the transgene expression cassette to enhance its therapeutic potential is adding specific peptides to generate chimeric enzymes with acquired capabilities. Lysosomal storage diseases (LSDs) are inherited metabolic conditions mostly caused by defective lysosomal hydrolases and often showing CNS involvement (40). The addition of heterologous signal peptides to soluble lysosomal enzymes has been showed to increase the secretion efficiency, thus improving enzyme bioavailability and tissue targeting upon *in vivo* GT in different models of LSDs, including MPS, GLD and Pompe diseases (41–44). In the case of Pompe disease, the liver directed administration of AAV encoding engineered secretable GAA (acid α-glucosidase) transgene in both mouse and non-human primate (NHP) animal models demonstrated improved efficacy associated to a clear dose advantage and reduced toxicity when compared to the native version of the GAA transgene. This approach is currently under clinical testing (NCT04093349). Furthermore, the fusion of the lysosomal hydrolase with specific protein domains capable to bind BBB receptors has been shown to allow active BBB crossing upon liver GT in preclinical LSD models. In these studies, the liver is converted into a factory for the engineered enzyme, which can cross the BBB and target the CNS upon secretion in the bloodstream (41, 45, 46). Interestingly, enzyme replacement therapy approaches based on the delivery of recombinant chimeric lysosomal enzyme fused to different BBB binding domains (BD) are under clinical evaluation for different MPS, thus supporting the potential clinical translation of GT protocols based on the viral mediated delivery of BBB-BD-modified enzymes.

An alternative way to enhance the therapeutic potential of the transgene is to use gain-of-function (GOF) mutants of the enzyme with increased activity and/or stability. Such "hyper functional" enzymes may be employed in *in vivo* gene transfer (as well as in enzyme replacement approaches) to produce a beneficial effect in targeted tissues at much lower doses and more efficiently compared to the respective WT enzymes. Naturally occurring GOF variants have already been used to treat liver diseases caused by inherited enzymatic defects. AAV vectors encoding a hyper-functional factor IX (FIX-Padua, R338L) has been explored for the treatment of hemophilia B. In dogs and mouse models of disease the use of such variant resulted in beneficial therapeutic effect and, at same time, allowed reducing the AAV vector dosage and, therefore, the risk of cellular immune response to vector capsid, which is one of the main complications of AAV GT for hemophilia B (18, 47, 48). In the case of lipoprotein lipase (LPL) deficiency (LPLD), an orphan disease associated with chylomicronemia, severe hypertriglyceridemia, metabolic complications, the use of AAV vectors encoding a GOF gene variant of LPL (S447X), showed efficacy in LPLD patients avoiding safety concerns related to immune response to AAV-capsid proteins (49). The possibility of generating GOF versions of enzymes "*ad hoc*" may greatly extend the possibility to apply safe GT protocols for the treatment of other metabolic diseases.

ENSURING DURABILITY OF LIVER GENE THERAPY FOR MONOGENIC DISEASES

Gene therapy for monogenic diseases promises to be a once-in-a-lifetime treatment that could be delivered at young age and last life-long. The clinical success obtained by AAV vector-based liver GT in adults with hemophilia has raised the expectation to extend enrollment to pediatric patients to maximize the potential benefits for the patients and to broaden the indications to diseases that are more severe or lethal in childhood, such as inherited diseased of liver metabolism. Because AAV vectors do not actively integrate into the host cell genome, they are progressively diluted upon cell division in liver growth, thus challenging their use in pediatric patients. To address this issue, AAV re-dosing, integrating vectors and genome editing and are being explored.

The anti-vector immune response induced after the first administration indeed currently limits the efficiency of a second administration, thus efforts are underway to counteract the anti-AAV immune responses and allow effective re-administrations (50–52). LV integrate into the target cell chromatin and are maintained as cells divide, thus being suited for stable

and potentially life-long transgene expression even following a single administration to newborn individuals. Systemic i.v. (intravenous) administration of LV has been shown to allow efficient and long-term gene transfer to the liver and achieve phenotypic correction of hemophilia in mice and dogs (53, 54). Allo-antigen free and phagocytosis-shielded LV have been generated, by high-level surface display of the phagocytosis inhibitor human CD47 (CD47hi) (55, 56). Following i.v. administration to NHP, these CD47hi LV provided amounts of circulating human coagulation factor transgene that would be therapeutic for hemophilia, the disease caused by the deficiency of one of these factors, without evidence of acute toxicity or genotoxicity. These LV are under development for clinical evaluation in hemophilia (57).

Site-specific integration of a corrective DNA in the genome remains an attractive therapeutic strategy for genetic diseases and represents an area of active investigation. The first report of successful *in vivo* genome editing in the liver in mice by ZFN dates back in 2011 by the K. High group, in collaboration with Sangamo Therapeutics (58, 59), an approach which has been later brought to early clinical testing in the context of Hunter's syndrome (60). The trial has been then closed and the results have not been published yet. In 2015, Barzel et al., reported a nuclease-free genome editing approach in the mouse liver, based on the spontaneous tendency of AAV vectors to integrate on a homology-dependent basis (61). This approach is being brought to early clinical testing in the context of the metabolic disease methyl malonic acidemia (https://investor.logicbio.com/news-releases/news-release-details/logicbio-therapeutics-announces-first-patient-dosed). Instead, Yin et al. reported in 2014 the first report of hepatocyte gene editing mediated by CRISPR/Cas9 for hereditary tyrosinemia type-I in mice (62). More recently, the advent of base editors has opened the possibility to perform single-base substitutions for therapeutic purposes (63). The availability of the highly efficient and transient LNP-based mRNA delivery system recently enabled nuclease-mediated or base-editor mediated genome editing in the liver of NHP and even humans (64, 65). Recently the results of the first clinical trial exploiting genome editing directed to the liver have been reported. These results showed high efficiency of gene disruption and evidence of therapeutic efficacy for the autosomal dominant disease TTR amyloidosis (22). The most advanced genome editing therapies remain so far mostly confined to gene subtraction approaches, however these encouraging results will fuel further progress toward more challenging gene correction approaches. Vector re-administration, integrating gene replacement and editing strategies have all advantages and disadvantages, thus extensive pre-clinical evaluations and risk/benefit assessments need to be conducted on an indication-per-indication basis.

ENHANCING THE DISTRIBUTION AND CELLULAR SELECTIVITY OF GTMPs TO IMPROVE *IN VIVO* CNS GT

The route of administration, the vector tropism, and the regulatory elements driving transgene expression are key determinants in defining the efficacy and safety of *in vivo* GT to treat CNS disorders.

In focal neurodegenerative disorders, intraparenchymal administration in the affected regions is well-tolerated and ensures a local distribution of the GTMP with low vector doses, thus reducing off-target effects in peripheral organs and immunogenicity (15). Convention enhanced delivery has been exploited to further increase the diffusion of the vector in the brain parenchyma by generating a pressure gradient in the infusion catheter leading to expansion of the extracellular space (66). The overall safety of intraparenchymal administration of AAV vectors has been shown in pediatric and adult patients affected by genetic (e.g., Canavan disease, Metachromatic Leukodystrophy, Batten's disease) and non-genetic (e.g., PD, AD) CNS disorders (66, 67). LV are alternative GT vehicles ensuring stable and robust expression of therapeutic transgenes in disease-bearing cells with negligible immune reactivity (68–72). The higher LV cargo capacity can be exploited to deliver multiple genes regulating metabolic processes that are hampered in genetic (i.e., GM2 gangliosidosis) (72) and sporadic (i.e., PD) diseases (70, 71). Indeed, the 8-year follow-up on ProSavin, a LV delivering key enzymes of the dopamine biosynthetic pathway, documented an improvement of the "off state" time in 8/15 treated PD patients, with GTMP-unrelated mild-to-moderate adverse events (16). Intrathecal or systemic administration can ensure a widespread biodistribution of viral vectors resulting in effective targeting of the spinal cord and in the rostro-caudal coverage of different brain regions (73). These approaches are better suited for the treatment of multifocal/diffuse neurodegenerative diseases (66, 67), including GM2 gangliosidosis (74). Still, they require higher vector doses and enhance targeting of off-target tissues, dorsal root ganglion pathology, and immune response against the GTMP (75, 76).

The selective delivery of GTMPs to the target cell populations/cell subtypes is necessary to improve both the efficacy and safety of GT. The efficiency of AAV vectors and LV in targeting different neuronal populations has been proven in rodents and NHP (15). The higher tropism of LVs for oligodendrocytes (69, 77–79), astrocytes (80) and microglia (81, 82) defines these vectors as a good candidate for gene transfer in glial populations. Recently, AAV hybrid serotypes and AAV variants generated by directed evolution or structural mutagenesis have been selected for their enhanced transduction efficiency in macroglia cells (83–85). In particular, systemic administration of the AAV9 variant AAV-F showed high proficiency for astrocyte transduction and a CNS distribution similar to the BBB-crossing AAV9.PHP.B variant (86), suggesting their potential use for less invasive targeting of cells involved in neuroinflammation processes.

The cell specificity of GTMPs could be enhanced by the inclusion of lineage-specific regulatory elements in the transgenic constructs. The size of cell-type specific promoters has been shortened to fit AAV cargo capacity and tested in pre-clinical models, resulting in upstream regulatory elements able to enhance and/or restrict transgene expression in neurons (e.g., NSE, CaMKII and Syn1 promoters) (87), astrocytes (e.g., gfaABC(1)D promoter) (84), oligodendrocytes (e.g., *Mag* promoter) (88), or microglia and brain-infiltrating macrophages (e.g., F4/80 and

CD68) (89, 90). De-targeting strategies based on endogenous microRNAs selectively expressed in off-target cell populations could further increase cell-specific transgene expression (81, 82), decrease the targeting of off-target cells/tissues (91, 92), and mitigate immune responses (93). The multiplexing of different microRNA de-targeting strategies favors the refinement of the post-translational regulation of transgene expression.

Nanoparticles (NPs) delivering large-size Cas9 nucleases, genome or epigenome modifiers are the ideal GTMP vehicle to ensure effective on-target editing by transient and safer expression of the editing machinery. Intraparenchymal injection of CRISPR/Cas9-loaded NPs have been tested in animal models to treat focal neurodegenerative disorders, such as Fragile X syndrome (94) and AD (95). The limited distribution and rapid clearance of NPs hamper their application in multifocal neurodegenerative diseases, for which multiple site administration or NP functionalization to increase cell-specific uptake and the BBB crossing are required to ensure CNS distribution upon systemic injection (96). Future *in vivo* validation of NP platforms to deliver GTMPs in the brain of large animals is a crucial step in the long path toward their clinical applications.

CONCLUSION

In vivo genetic engineering has experienced considerable progress in the last decade and a few landmark studies have convincingly shown that somatic genetic modification for therapeutic purposes can be safely achieved in humans. These new advanced therapies remain highly complex, only partially understood, difficult and costly to develop. Yet, they hold tremendous therapeutic potential and promise to revolutionize medicine. We have highlighted some of the many challenges that still need to be addressed and some avenues that are being explored for broader exploitation and effective introduction of these therapies into clinical practice. To achieve this goal, technical advances need to be accompanied by a continuous dialogue and cooperation between academia, biotechnology and pharmaceutical companies, regulators, policy makers and a civil society with high education and trust in science.

AUTHOR CONTRIBUTIONS

AC, AF, VM, and AG: wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Italian Ministry of Health (GR-2019-12368956) to AC; Cure Sanfilippo Foundation and Sanfilippo Children's Foundation (Joint grant 2021-2023), Fondazione Telethon (Tigem Core grant 2016-2020) to AF; Fondazione Telethon (Tiget Core Grant 2016-2021, grant D2), European Leukodystrophy Association (ELA 2019-015I2), European Joint Program on Rare Diseases (EJPRD; project NG4Leuko) to AG; EU Marie Curie fellowship (895111 ASTRO-EDITING), Italian Ministry of Health (GR-2019-12368930), ELA (ELA 2020-010I2) to VM.

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frontiers in Medicine

PERSPECTIVE
published: 03 February 2022
doi: 10.3389/fmed.2022.757647

Advanced Therapy Medicinal Products' Translation in Europe: A Developers' Perspective

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Advanced Therapy Medicinal Products (ATMPs) comprising cell, gene, and tissue-engineered therapies have demonstrated enormous therapeutic benefits. However, their development is complex to be managed efficiently within currently existing regulatory frameworks. Legislation and regulation requirements for ATMPs must strike a balance between the patient safety while promoting innovations to optimize exploitation of these novel therapeutics. This paradox highlights the importance of on-going dynamic dialogue between all stakeholders and regulatory science to facilitate the development of pragmatic ATMP regulatory guidelines.

Keywords: regulatory affairs, European Medicines Agency, legislation, regulatory science, Paul-Ehrlich-Institute (PEI), advanced therapy medicinal product (ATMP), cell and gene therapies

OPEN ACCESS

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *12 August 2021*
Accepted: *10 January 2022*
Published: *03 February 2022*

Citation:
Pizevska M, Kaeda J, Fritsche E, Elazaly H, Reinke P and Amini L (2022) Advanced Therapy Medicinal Products' Translation in Europe: A Developers' Perspective. Front. Med. 9:757647. doi: 10.3389/fmed.2022.757647

INTRODUCTION

Navigating an Investigational Medicinal Product (IMP) through the regulatory maze to the clinic is time-consuming and expensive, with many stakeholders involved. New therapies must be rigorously tested *in-vitro* and subjected to exhaustive pre-clinical investigations in accordance with regulatory guidelines to ensure they are safe and supposedly efficacious prior to clinical trials. The existing regulatory frameworks are proving cumbersome especially when it comes to implementing first-in-human (FIH) developments and do not sufficiently reflect the great heterogeneity of the novel Advanced Therapy Medicinal Products (ATMPs). The ATMPs are logistically challenging, have complex manufacturing procedures, demanding approval processes, are highly individualized and as a consequence exceptionally expensive (1). However, ATMPs have the potential to eliminate or repair disease causing cells, offering a curative approach with opportunities to address unmet medical needs (2, 3) and the opportunity for highly personalized precision medicine. To date the majority of the currently approved ATMPs target orphan disease indications (4), but are advancing at pace such that regulatory authorities and the developers must adapt the assessment procedures and the legislation without compromising patient safety and hampering innovations (5). As such, these novel products exhibit a variety of unique characteristics that are challenging to the traditional health care systems leading to limited access by patients and, in some instances, market discontinuation (6). Global efforts are underway to improve the economic value of ATMPs by improving methods of manufacturing and adapt them for scaling (7) and advance the necessary infrastructure to treat monogenic and rare diseases (8). It is incumbent upon stakeholders to develop new tools, standards, and approaches to assess the safety, efficacy, quality, and performance of the novel pharmaceutical products (5). These tools are integral to the principles of regulatory science which ensures that data-driven policies are in place to facilitate safe and timely availability

of life-saving medicines (8). Most importantly, for ATMPs to be widely available to patients worldwide, harmonizing regulatory convergence among countries should now become a priority more than ever (9), an important lesson the scientific community learned from the COVID-19 pandemic. This appraisal highlights the challenges facing regulatory science to foster science-based decision making into safeguarding public health and promoting innovation.

THE CURRENT REGULATORY FRAMEWORK

In 2009 following the implementation of the Regulation 1394/2007 (10), and recognizing the innovative characteristics of ATMPs, a multidisciplinary expert committee within the European Medicines Agency (EMA), the Committee for Advanced Therapies (CAT) was established. To enable a European-wide market access, the centralized procedure on marketing authorization application (MAA) for ATMPs became mandatory, benefitting from a single evaluation process (11, 12). Additionally, in 2016 EMA launched a PRIority MEdicines (PRIME) scheme to enhance fast track development of medicines that target an unmet medical need and thereby ensure faster patient access. This accelerated pathway provides active support to efficiently develop agents for unmet medical needs and does not require large datasets. This is counterbalanced by a need for more stringent post-market safety and efficacy evaluations (13).

The clinical trial approval, evaluation and monitoring however is devolved to the individual EU member states (MS). For example, ATMP regulations in Germany are especially exacting, requiring (a) clinical trials authorization from the national competent authority (NCA) "Paul-Ehrlich Institut" (PEI); (b) approval from the local ethics committee within the state the principle investigator is located, and (c) manufacturing license authorization from the respective local competent authority ("Landesbehörde") (14). Furthermore, the collection of starting material, e.g. peripheral blood, is subject to the German Transfusion Act (Transfusionsgesetz; TFG) (15) and/or German Transplantation legislation (16), while the local authority must approve the tissue collection site ("Entnahmeinrichtung"). If the product is considered a genetically modified organism (GMO), the PEI is responsible for environmental risk assessment in consultation with the Federal Office for Consumer Protection and Food Safety ("Bundesamt für Verbrauchschutz und Lebensmittelsicherheit") (17).

Consequently, delays due to the variations in GMO regulation across MS result in a less-competitive and less-attractive environment for stakeholders to realize multicenter clinical trials with investigational gene therapies in Europe and has been issued by multiple stakeholders (18). Together with national competent authorities, they demand to exempt ATMPs containing or consisting of GMOs from the GMO legislation, as it has been temporarily adopted by the EU for IMPs treating or preventing COVID-19 in human (19). This exemption for IMPs to treat or prevent COVID-19 had timely and administrative benefits for the sponsors and trial sites. Stakeholders and advocates of ATMPs expect a rapid implementation of a GMO exemption scheme under the pretext of the new Clinical Trial Regulation (EU) No 536/2014 (20), which will come into force January 2022.

NON-CLINICAL REGULATORY REQUIREMENTS FOR ATMPs

Given the heterogeneity and the complexity of ATMPs, which frequently involve viable cells ("living drug"), the conventional strategies designed for robust non-clinical (NC) assessment of proof-of-concept (PoC), mechanism of action (MoA), toxicology and bio-distribution are not always transferable to ATMP development. Standard non-clinical murine, *ex-vivo* assessment of dose-related safety and efficacy to test ATMPs have limited value specifically, due to the differing immunologic background and microenvironments. Elsallab et al. noted ATMPs have the disadvantage of significant uncertainties with NC translation data which may influence their benefit risk assessment. This is due to several factors, including lack of relevant animal models and clear primary pharmacological targets. Therefore, a major challenge is to identify platforms enabling rigorous evaluation of NC outcomes, which are meaningful and predictive for human clinical trials (21). To overcome these hurdles developers need to foster collaborations with industry partners and engage with regulatory agencies to define, evaluate and develop appropriate NC models where relevant data is unavailable.

MANUFACTURING ATMPs

It is mandatory that ATMP manufacturing complies with good manufacturing practice (GMP) guidelines, which includes using GMP grade starting materials. But frequently, GMP grade starting material is scant and expensive. The lack of standardized regulatory framework tailored to small-scale production and for establishing specific pharmacopeia monographs for pharmaceutical grade raw materials and raw materials of biologic/human sources leads to fragmented manufacturing and impacts on quality, precision, purity, functionality, reproducibility, and stability. These challenges are often compounded by the lack of adequate expertise, technical equipment and trained personnel specific to ATMP GMP compliance. Furthermore, ATMPs are often designed for a small specific group of patients or are highly individualized. As a consequence their manufacturing is not easily amenable to GMP compliance nor automation to enable commercialization at viable cost-effective levels (22). Moreover, GMP as well as quality control guidance specific to ATMPs often lack precise details or are not suitable. For example, the guidelines EMA/CAT/80183/2014 (23) and EMA/CAT/GTWP/671639/2008 (24) include phrases such as "unless otherwise justified" or "case-by-case basis," which leaves both the NCAs and the developers in disparity in how to interpret the legislation and implement GMP compliant strategies. Consequently, the regulatory agencies, NCA and the developers face a quandary in how to achieve balance between flexibility while aiming to provide clarity. We suggest

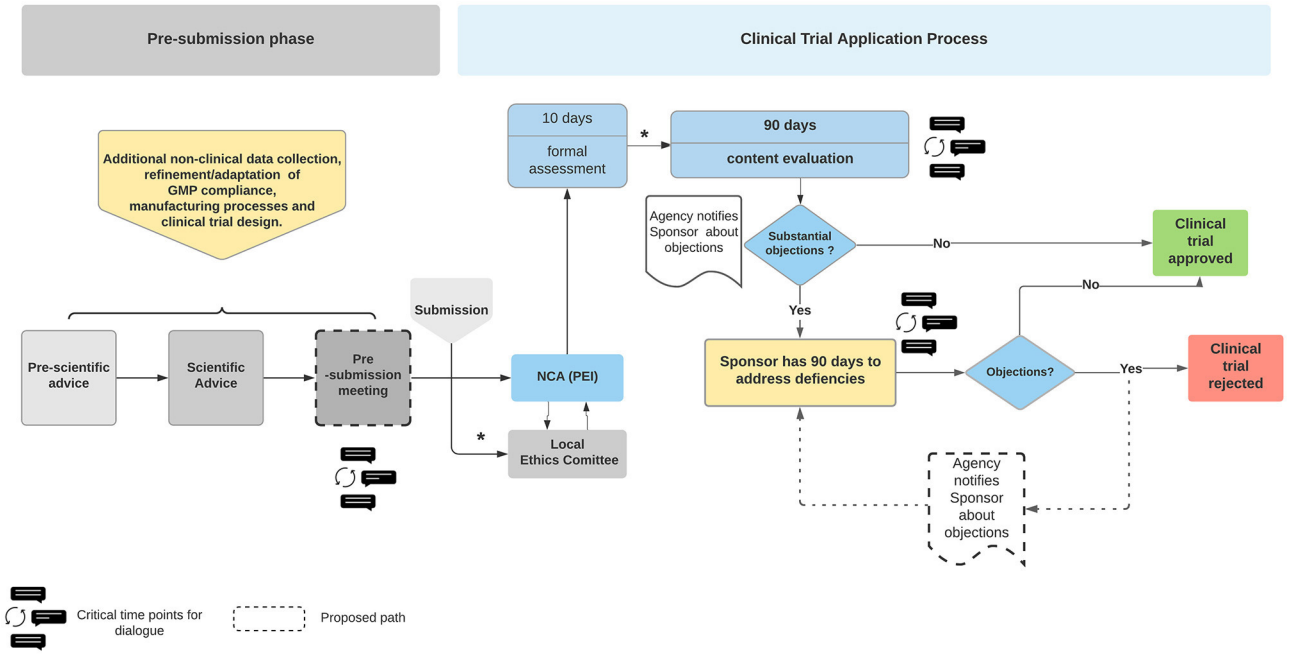

FIGURE 1 | Overview of the Clinical Trial Application process for ATMPs in Germany. The Sponsor has opportunity to seek advice from the National Competent Authority (NCA), Paul Ehrlich Institute (PEI). The Sponsor is notified of the regulators' concerns at the first review discussions following formal submission. The Sponsor has 90 days to address the issues raised. The clinical trial is rejected if the regulatory body is not satisfied with the responses. The dialogue bubbles indicate the proposed timepoints for a dialogue between the regulators and sponsors. The process describes the current legislative process. The timelines may differ once the Clinical Trial Regulation EU No 536/2014 is enacted in January 2022. The boxes with dashed lines are Authors' suggestions in how to create a more dynamic dialogue, optimizing the outcome in favor of safer therapies which are available more rapidly. Blue boxes, PEI related actions; Yellow boxes, sponsor related actions. *Communication between Ethics Committee and Sponsor and intermediate steps regarding formal assessment during the CTA process are not depicted (not relevant in this context). NCA, national competent authority.

this impasse could be circumvented by intensive interactive discussions throughout e.g., the early development process (before FIH application) involving experts during the CTA review process (see **Figure 1**).

As the GMP governance is entrusted to individual EU member states, the NCAs may request additional information, thus introducing another variance in applying ATMP pharmaceutical quality control across borders. In Germany, with the federal structure, the local competent authority governs GMP and grants manufacturing licenses in accordance with section 13 of the Medicinal Products Act (Ger. AMG) by the respective authority (16 in Germany) of the Federal State (Ger. *Länderbehörde*), where the manufacturing site is located (25).

To address these concerns the EudraLex Volume 4 Part IV advanced the framework for GMP-compliant ATMP manufacturing (26). While providing invaluable information and flexibility, to be applied to different cases/products of the ATMP repertoire, the built-in flexibility means the guidelines are open to interpretation and misunderstanding, which may lead to the failure to achieve the required quality standards.

QUALITY CONTROL

Quality control of ATMPs is especially complex, as they require sophisticated testing in comparison to chemical compounds, for example genetically modified cell products which are expected to bring additional potential risks to patients. In this regard, the specificity and safety of genetic modification need to be carefully examined to eliminate the risk of malignant transformation and off-target effects. These concerns were highlighted by a gene therapy trial to treat children with X-linked severe combined immunodeficiency. Some of the patients developed acute lymphoblastic T-cell leukemia following gene therapy, due to vector-mediated insertional mutagenesis (27, 28). The governance of novel technologies such as designer nucleases, e.g., CRISPR/Cas9 technology, require the development of advanced strategies to identify potential complications such as off-target editing or immunogenicity. It is challenging to definitively assess off-target and long-term effects when manipulating genes or administering genetically modified cells which can differentiate and evolve in response to surrounding stimuli (29). To their credit, the EMA's Committee for Advanced Therapies (CAT) acknowledges the need to develop regulatory guidelines covering quality, safety, and efficacy that are relevant to ATMP. Therefore, CAT proposes guidelines and opens them up for public consultation. Equally, it is being acknowledged that while *ex-vivo* gene-editing strategies may be similar, *in-vivo* gene editing requires new regulatory rules for quality, safety and efficacy testing (29). In order to realize such changes, discussions at national and EU level between the developers and regulatory authorities are needed.

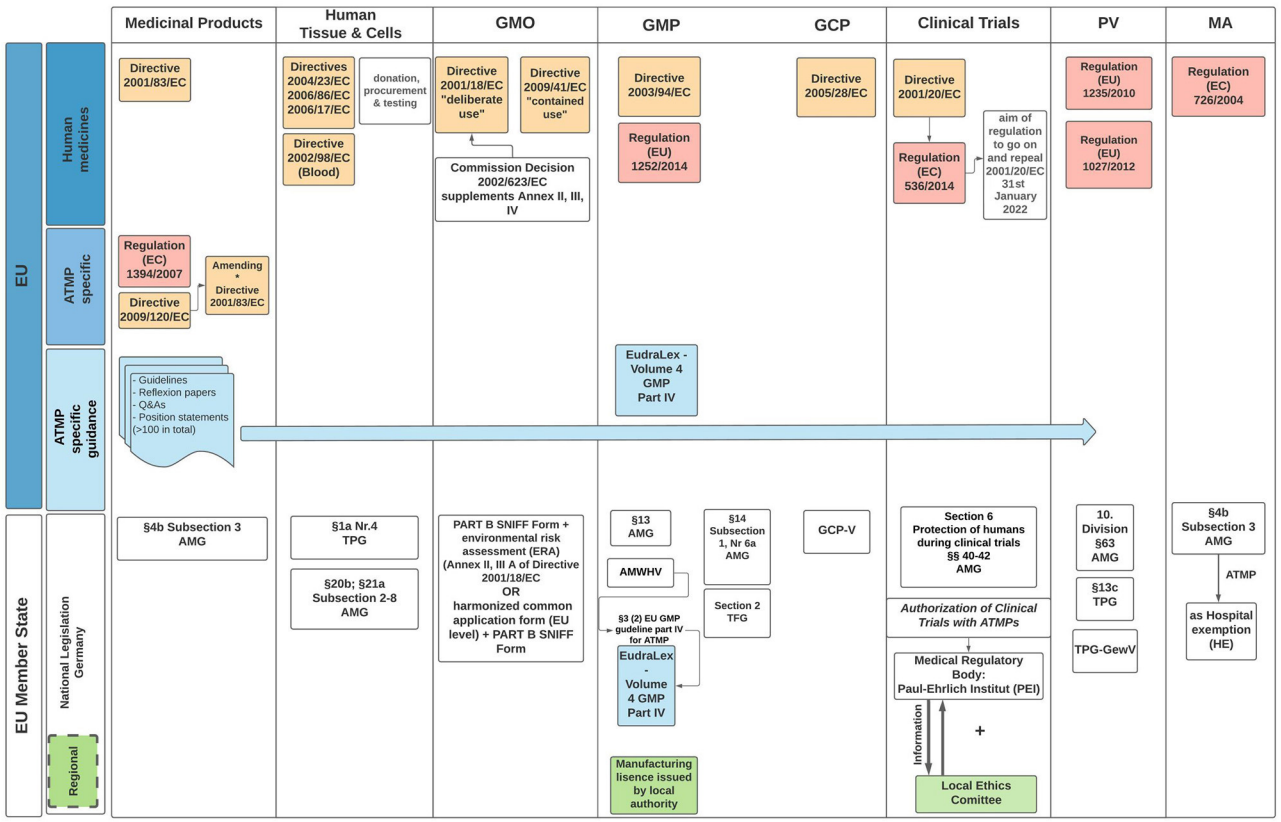

FIGURE 2 | EU and German regulatory framework for ATMP specific legislation. The diagram outlines the directives EU Member States (MS) must enact, with particular reference to the regulatory guidelines applicable when seeking IMP authorization in Germany. Governance and oversight of clinical trials is the responsibility of the MS undertaking the trial. In Germany, regional authorities of the federal states are responsible for issuing a manufacturing approval. Clinical Trial authorization takes place with agreement of the local Ethics Committee. AMG, arzneimittelgesetz (*Engl.* German drug law); TPG, transplantationsgesetz (*Engl*. transplantation law); TPG-GewV, TPG—Gewebeverordnung (*Engl.* tissue regulation); TFG, transfusionsgesetz (*Engl.* Transfusion law); AMWHV, arzneimittel- und wirkstoffherstellungsverordnung (*Engl*. ordinance for the manufacture of medicinal products and active pharmaceutical ingredients); GMO, genetically modified organism (includes medicinal products with GMOs); GMP, good manufacturing practice; GCP, good clinical practice; PV, pharmacovigilance; MA, marketing authorization; Red boxes, regulations; Yellow boxes, directives; Green boxes, regional; Blue boxes, regulatory framework applicable to all EU MS.

BENEFIT-RISK ASSESSMENT CHALLENGES

Unlike the traditional drugs, the novel ATMPs are frequently not or cannot be tested in healthy human volunteers as is the case in the classical phase I study. ATMP trials usually are FIH studies combined as phase I/IIa and directly enroll critically ill patients. Furthermore, often only a small number of patients are included in clinical trials. Equally it is generally accepted that potential toxicities cannot be adequately addressed for ATMPs (30). Indeed, given the nature of ATMPs, benefit-risk assessment is not easily defined or measured because side effects depend on a variety of factors that are difficult to model in NC experiments. Equally, potential toxicities are rarely if at all detectable in the NC studies that are performed (31). Moreover, the availability of safety data in both, the non-clinical and clinical part is limited. Therefore the commonly recommended appropriate risk mitigation measures are especially important in ATMP clinical trials. These measures include, exceptionally close monitoring, rapidly accessible treatment options with intensive care units in close vicinity and fully trained medical professionals must be available at the trial site. Where data from animal models are available, they must be evaluated and safety data extrapolated to a FIH trial if necessary. Therefore, as mentioned above, the exceptional circumstances of ATMP development require close and regular communications in early stages of review processes between the regulatory agencies and the developers. These discussions are critical in tailoring the clinical trial accordingly (**Figure 1**). Experience and the relevant data gleaned from such exchanges can be chelated to formulate fit-for-purpose regulations. In this context, and as a result of a constantly changing knowledge base with time, the so called "adaptive governance" is in discussion (32), even more important since the COVID-19 pandemic. However, any novel fast access tools/mechanisms might raise concerns about the integrity of the data. As demonstrated by the public's anxiety about the

speed with which SARS-CoV2 vaccines were authorized. These concerns are being addressed by longer follow-up periods and implementation of extensive post-marketing authorization studies (post authorization safety and efficacy studies). Such an approach also addresses the uncertainties about products' benefit-risk balance at the time of marketing authorization (13). Indeed, the post market authorization and approach to assess safety and efficacy in lieu of traditional randomized clinical trials is being formally explored by FDA (33, 34), commonly referred to as Real World Evidence (RWE) by analyzing Real World Data (RWD), see below.

REGULATORY SCIENCE

Regulatory science encompasses basic and applied biomedical as well as social sciences, and contributes to the development of regulatory standards and tools (35). Stakeholders within regulatory science recognize the inherent challenges in drug development and aim to bridge the gaps in the technical, regulatory, reimbursement and health technology assessment (HTA) knowledge. Such an approach is expected to enable formulation of regulations that lead to science based decision-making processes and thereby improve ATMP development and efficacy (36).

In consultation with the stakeholders, EMA refined their strategy and included a number of recommendations in the "Regulatory Science to 2025 strategy" (37). This comprises (a) establishing a multi-stakeholder forum to foster innovation in clinical trials; (b) re-enforcing relevance of patients for evidence generation; (c) promoting the use of real-world data and big data in decision-making processes; (d) providing a feasible legislative framework as well; (e) contributing to a better HTAs' preparedness and decision-making at national levels to foster innovative medicines development while enhancing translational dialogue with payers to enhance accessibility.

More recently, the STARS (**S**trengthening **T**raining of **A**cademia in **R**egulatory Science) consortium comprising 18 European regulatory agencies including EMA was established to strengthen the bidirectional dialogue between research scientists and regulatory bodies. STARS seeks to address the challenges listed above in ATMP development by first taking an inventory of the current support structures for regulatory scientific advice in academic institutions and gathering feedback on their needs. The goal is to develop a common strategy for scientific advice to be implemented by the relevant national authorities (38, 39). By establishing initiatives like STARS it provides a forum for discourse to adapt and evolve new practices and insights with the expectations that are workable and effective guidelines can be standardized across EU MS and simultaneously evolve training practices for researchers in regulatory science.

Because academia is often at the forefront in developing novel ATMP therapeutics, their input is critical in any regulatory science discussions. The academia research institutions can assist in driving the agenda *via* translational hubs as is the case in UK exemplified by "Advanced Therapy Treatment Centres" (40) and in some of the EU MS [e.g., (41–43)], but which are relatively scant in Germany. Hence, the German Research Foundation (DFG) is seeking to create an environment in which inter-medical university infrastructures across Germany can be established. Translational hubs provide an opportunity platform platform for cross fertilization of public and private institutions to advance ATMP therapeutics and make regulatory science based recommendations to committees such as CAT. In addition to academic hubs, there are examples of independent centers of excellence for example "CATAPULT—Cell and gene therapy" in the UK, which work at the interface between commercial enterprises and academia (44).

The advantages of translational hubs is exemplified by the RESTORE and ReSHAPE consortia (45, 46), which led to the development and translation of ground-breaking cellular therapies, including T cells, to modulate the immune systems in living donor transplant recipients enabling reduction of dependency upon toxic immunosuppressive drugs (47, 48). However, this also required development of new GMP compliant procedures through frequent discussions in how current regulatory guidelines should be applied and adapted where necessary.

There is a need for bi-directional discussions among all stakeholders especially during pre- and post-submission of a clinical trial application (CTA), especially when considering FIH studies (**Figure 1**). These exchanges would provide the applicant with an opportunity to clarify any ambiguities and identify solutions to unforeseen difficulties. Indeed, the USA Food and Drug Administration's (FDA) (49) guidance document provides a forum for the applicant to engage with the regulatory agencies to ask questions about the specific requirements and clarify any misunderstandings. These FDA-Developer discussions take place prior to submitting a final response during the CTA process, thus diminishing the possibility of approval for the trial being denied. An analysis conducted by the Alliance for Regenerative Medicine (ARM) regarding clinical trials for ATMPs in Europe supports the aforementioned concept by showing that a scientific advice prior CTA increases the speed of CT approval and decreases the questions raised by the regulatory bodies. According to this survey, the most important criteria for selecting a clinical trial site and country are the expertise of the health care professionals, quality of review and the expertise of regulatory authorities (50).

DISCUSSION

Heterogeneous ATMPs continue to evolve at a rapid pace, providing options for unmet clinical needs. However, the traditional approach to conducting clinical trials is not directly applicable to ATMPs requiring a change in culture. The ATMP-specific legislation is ambiguous in terms of exact requirements as highlighted above.

ATMP legislation poses a dilemma in trying to balance innovative therapies requiring flexibility and provide detailed, well-defined legislation. Regulatory agencies and investigators acknowledge, ATMP oversight is obdurate as several regulatory frameworks must be considered in parallel when developing these products (**Figure 2**). Formulation of acceptable regulations within the EU is further complicated by the additional layer of national legislation (51). Stakeholders through translational hubs need to coalesce to define new standards with the aim of developing fit-for-purpose ATMP regulatory guidelines. Integral to this process is regulatory science as a competency within academia that could advice, formulate, and scrutinize innovative ATMP therapies. Failure to address harmonization concerns within Europe will lead to loss of expertise and innovation to USA, UK, China, and Asia more widely.

However, in the rapid changing field of ATMP stakeholders need to be supported by government finance and governance. A proactive approach by the authorities led to the development and approval of SARS-CoV2 vaccine at an unprecedented speed, without compromising patient safety. Shifting scientific advice meetings to take place online would facilitate the availability of appropriate worldwide expertise during approval discussions between stakeholders and thereby overcome ATMP development associated complexities.

Limited availability of clinical data means risk/benefit assessment is challenging. This could be addressed by gathering RWD, i.e., gathering data from numerous sources, e.g., electronic health records, medical data bases and patient information in post authorization studies. Such information provides RWE for ATMP clinical trials where the traditional randomized controlled large scale trials are not feasible or not applicable, e.g., patient population (52–57). The RWD from anti-CD19 CAR T cell therapy have demonstrated that in post marketing stages patients are much more advanced in disease, heterogeneous and the manufacturing period is longer than in the tightly controlled clinical trial setting (58, 59). The EU Commission also proposes to revise the current pharmaceutical legislative to include "new methods of evidence generation and assessment" (60).

This perspective has sought to highlight the considerable challenges stakeholders face in balancing the therapeutic potential of novel treatments while maintaining regulatory standards which are evidence based and designed to ensure patient safety. This distinction is not always absolute, requiring continuous exchange of available options with independent scientific expert advisors assisting to eliminate any ambiguity/discrepancies. Because innovations in therapeutics will continue to challenge the guidelines. The legislation must co-develop with the ATMP evolution in order to ensure the translation of innovative therapies. Support of regulatory science in the scientific field and close interaction with legislative bodies will create an environment which is more specifically tailored to the rapidly evolving needs of ATMP development to ensure more efficient market penetration for the benefit of patients.

GENERAL RECOMMENDATIONS

- Encouraging effective networking between academia, industry, patient initiatives and other stakeholders.
- Emphasize and implement regulatory science as a specialized discipline.
- Support translational hubs, consortia and other structures to facilitate in bringing ATMPs to the clinic.
- Early engagement and bidirectional dialogue with the regulatory authorities.
- Enhance international harmonization efforts on ATMP legislation between EU MS and beyond.
- Recognize translation of ATMPs as a collaboration effort between all stakeholders (scientists, physicians, industry, patients AND regulatory agencies).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MP, JK, EF, HE, and LA undertook literature research and reviewed and wrote the manuscript. MP designed the Figures. PR provided the funding and reviewed the final draft. All authors contributed to the article and approved the submitted version.

FUNDING

The project was partially funded from the European Union's Horizon 2020 research and innovation program under grant agreement No. 820292 (Restore) and No. 825392 (Reshape).

ACKNOWLEDGMENTS

Authors would like to thank Professor Hans-Dieter Volk [Berlin Institute of Health (BIH) Center for Regenerative Therapies (BCRT) and Berlin Center for Advanced Therapies (BeCAT), Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin] for his invaluable support and for reviewing the manuscript.

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frontiers | Frontiers in Medicine

PERSPECTIVE
published: 06 June 2022
doi: 10.3389/fmed.2022.913287

Toward Rapid, Widely Available Autologous CAR-T Cell Therapy – Artificial Intelligence and Automation Enabling the Smart Manufacturing Hospital

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OPEN ACCESS

Edited by:
Hans-Dieter Volk, Charité Medical University of Berlin, Germany

Reviewed by:
Guido Moll, Charité Universitätsmedizin Berlin, Germany

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Specialty section:
This article was submitted to Translational Medicine, a section of the journal Frontiers in Medicine

Received: *05 April 2022*
Accepted: *11 May 2022*
Published: *06 June 2022*

Citation:
Hort S, Herbst L, Bäckel N, Erkens F, Niessing B, Frye M, König N, Papantoniou I, Hudecek M, Jacobs JJL and Schmitt RH (2022) Toward Rapid, Widely Available Autologous CAR-T Cell Therapy – Artificial Intelligence and Automation Enabling the Smart Manufacturing Hospital. Front. Med. 9:913287. doi: 10.3389/fmed.2022.913287

CAR-T cell therapy is a promising treatment for acute leukemia and lymphoma. CAR-T cell therapies take a pioneering role in autologous gene therapy with three EMA-approved products. However, the chance of clinical success remains relatively low as the applicability of CAR-T cell therapy suffers from long, labor-intensive manufacturing and a lack of comprehensive insight into the bioprocess. This leads to high manufacturing costs and limited clinical success, preventing the widespread use of CAR-T cell therapies. New manufacturing approaches are needed to lower costs to improve manufacturing capacity and shorten provision times. Semi-automated devices such as the Miltenyi Prodigy® were developed to reduce hands-on production time. However, these devices are not equipped with the process analytical technology necessary to fully characterize and control the process. An automated AI-driven CAR-T cell manufacturing platform in smart manufacturing hospitals (SMH) is being developed to address these challenges. Automation will increase the cost-effectiveness and robustness of manufacturing. Using Artificial Intelligence (AI) to interpret the data collected on the platform will provide valuable process insights and drive decisions for process optimization. The smart integration of automated CAR-T cell manufacturing platforms into hospitals enables the independent manufacture of autologous CAR-T cell products. In this perspective, we will be discussing current challenges and opportunities of the patient-specific but highly automated, AI-enabled CAR-T cell manufacturing. A first automation concept will be shown, including a system architecture based on current Industry 4.0 approaches for AI integration.

Keywords: ATMP, CAR-T cell, artificial intelligence, automation, autologous, manufacturing, Industry 4.0, smart manufacturing hospital

INTRODUCTION

With the transformation of hospitals toward smart treatment centers, digitalization is entering the health sector and supporting hospital employees through intuitive digital data management and robotics. Successes have already been achieved in diagnostics, treatments, and surgical intervention. In recent years, Advanced Therapy Medicinal Products (ATMPs) have gained importance for curing genetic and cellular diseases. One therapy already being applied to treat acute leukemia and lymphoma is CAR-T cell therapy. In contrast to traditional cancer treatments, CAR-T cell therapy allows for the specific targeting of tumor cells. The approved therapies Kymriah®, Yescarta®, and Tecartus® (1–3) target the CD19 antigen in hematological malignancies but differ in cell composition, manufacturing process, and costimulatory domain. These therapies use an autologous approach, where the patient's cells are engineered instead of allogeneic cell therapies where cells are extracted from a healthy donor, engineered, and expanded to treat multiple other patients. Allogeneic CAR-T cells offer the opportunity for large-scale production. However, they cause significant graft-vs.-host disease and are rapidly terminated by the host's immune system, currently limiting their applicability (4). Autologous therapies have seen clinical approval but face manufacturing and large-scale deployment challenges. **Figure 1** visualizes these challenges along the six main steps of CAR-T cell therapy.

The entire process from provision of starting material (e.g., apheresis, blood donation) to injection is currently dominated by **impractical manual processes**. These processes are highly complex, requiring much personnel and generating high costs due to their labor-intensiveness, cost of materials, and use of large cleanroom suites. Additionally, manual manufacturing leads to frequent interaction of personnel and product, increasing the risk for contamination and subsequent product loss. A transition away from these manual and static manufacturing protocols is needed to shorten production cycles to improve vein-to-vein timelines. As autologous therapies are keyed to an individual patient, the current centralized production increases overall manufacturing times and generates **avoidable logistics** due to laborious transportation of apheresis, viral vectors, and CAR-T cell product. Compared to the established therapies the vein-to-vein timelines [e.g., 17 days for Yescarta (5)] can be reduced and consequently the patient's chances of recovery increased.

Closed, semi-automated systems have been developed to address these issues, such as the Miltenyi Prodigy® and the Lonza Cocoon® (6, 7). These devices follow a "one-device-per-patient approach" to minimize the risk of cross-contamination. Unfortunately, this manufacturing approach is unsuited for large-scale deployment, limiting the reduction of manufacturing costs and widespread application of CAR-T cell therapy. These devices are time-consuming to adapt to technological advances in the field due to their high level of integration and technological

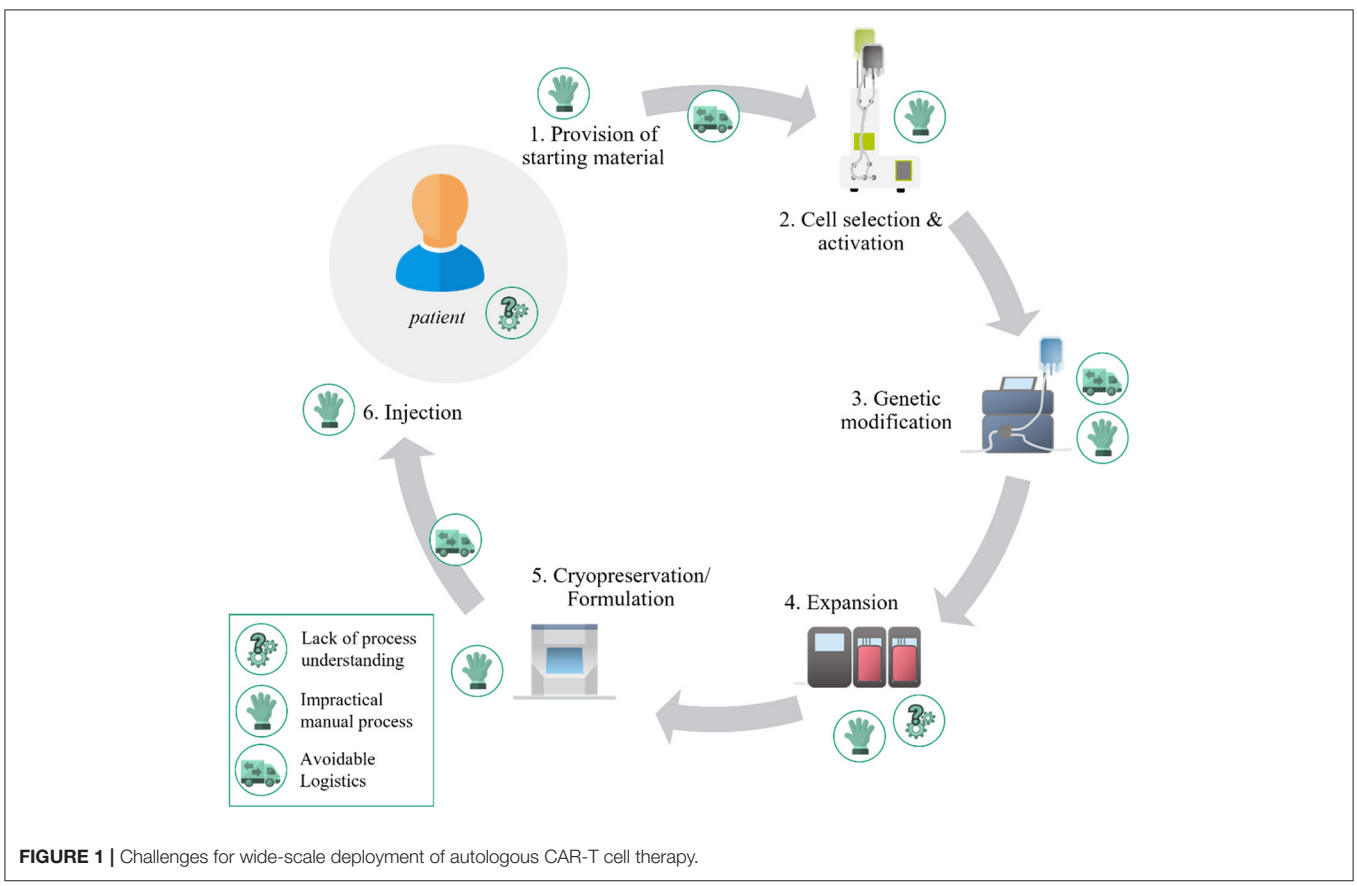

FIGURE 1 | Challenges for wide-scale deployment of autologous CAR-T cell therapy.

complexity. Additionally, these devices do not provide the necessary process insights to assess cell quality and provide early information on the performance of the cells and potential therapeutic outcome.

Installing more process analytical technology to generate a broad data basis combined with data analytics and AI approaches is needed to overcome a **lack of process understanding**. Even if regulatory hurdles often still have to be overcome, AI is already being used successfully in the hospital context (8). McKinsey created an overview of the AI solutions currently used in Europe and classified them according to a patient-centered healthcare framework (9). Most of the identified use cases are in diagnostics and clinical decision making, whereas a typical use case in diagnostics is the automated counting of living and dead cells in a blood sample. CAR-T cell therapy can highly benefit from the solutions already in use and the overall potential of AI.

A novel automated manufacturing approach is needed to treat high numbers of patients with autologous CAR-T cell therapies at the state-of-the-art. This automated system should allow for parallelized production of autologous CAR-T cell products to decrease costs and increase the product's availability. It needs to be designed in an integrated but modular manner to allow for rapid adaption to technological advancements in the field of ATMP manufacturing but also decreases hands-on interventions to a minimum.

The automation and AI integration require the second transformation of hospitals into smart manufacturing hospitals by enabling them to produce CAR-T cells directly at the point of care. A **smart manufacturing hospital** is defined as a hospital specialized on ATMPs that incorporates an end-to-end automated manufacturing platform for personalized treatment in an adjacent GMP facility. The facility connects to existing logistic and IT infrastructure, offering extensive patient and manufacturing data availability as well as AI-driven clinical decision support while taking all regulations (e.g., G(A)MP, MDR, cybersecurity) into account. For a flexible and modular integration of the manufacturing platform into the GMP facility and the hospital, an IT infrastructure based on reliable Industry 4.0 and IIoT (Industrial Internet of Things) is needed to cope with the rapidly changing environment of automated cell and gene therapy. Existing approaches (10–12) already show applicability to the hospital context but do not meet the new demands of the smart manufacturing hospital. In particular, the reliable provision of patient and manufacturing data and comprehensible decision support for the manufacturing process requires a novel, holistic approach to exploit the full potential of automated CAR-T cell therapy.

AUTOMATED AI-DRIVEN CAR-T CELL MANUFACTURING

For decentralized onsite manufacturing of ATMPs, the hospital infrastructure needs to be adapted. As such, a manufacturing platform should operate mostly independent of highly trained personnel producing autologous CAR-T cells autonomously. The deployment of an automated AI-driven CAR-T cell manufacturing platform requires extensive knowledge of the underlying biological process, hardware (e.g., devices, machines), and software components (e.g., control software, data management, AI models). Since the three areas are highly interconnected, a close interdisciplinary exchange of all stakeholders is essential for success. Our automated AI-driven CAR-T manufacturing concept, developed within the scope of EU H2020 project AIDPATH (AI-driven, Decentralized Production for Advanced Therapies in the Hospital) (13, 14), focuses on these three areas and approaches the technological challenges and potential solutions. **Figure 2** gives an overview of the manufacturing platform executing the CAR-T cell process and the software components enabling process control, AI integration, and data management.

The **CAR-T cell process** poses several challenges toward automation. Firstly, as an ATMP production process, it must be conducted under aseptic conditions and according to Good Manufacturing Practice (GMP) (15). Maintaining large cleanroom suites in a hospital for ATMP manufacturing is impractical, therefore aseptic production in cleanrooms with lower grade and smaller footprints is essential. Secondly, for sustained use of the automated manufacturing platform in CAR-T cell manufacture, it should accommodate variations in bioprocess set-up and design. Each step in CAR-T cell manufacturing may differ dependent on the kind of CAR-T cell therapy to be produced. As a hospital treats many different patients with different needs, the platform has to be suited to a multitude of ATMP products and allow for easy implementation of new ones (16). For instance, while genetic engineering has typically been achieved by viral transduction, more and more processes utilize non-viral transfection methods to transfer the CAR-DNA (17, 18). Therefore, a universally acceptable manufacturing platform should accommodate both viral and non-viral methods. Studies have also shown the efficacy of therapies with both $CD4^+$ and $CD8^+$ CAR-T cells (19). Currently, this is achieved by culturing both cell types separately and then combining them for the formulation of the therapy. To improve treatment efficacy without increasing process burden, a co-cultivation of $CD4^+$ and $CD8^+$ T cell populations is preferable to eliminate the need for two separate cell cultures running in parallel. Lastly, while some CAR-T cell therapies are cryopreserved before injection, some are held until product release and then directly transferred to the patient without prior freezing (20). Additionally, cryopeservation can thereby have a high impact on the outcome of the therapy (21). All of these different modes of operation need to be represented by the different hardware required for each individual process step, but also by a highly flexible software architecture allowing for these adaptions to different CAR-T products.

To automate autologous CAR-T cell therapy manufacturing, all required **hardware**, such as machines and devices, need to be combined in one integrated process pipeline. As depicted in **Figure 2** the manufacturing plant consists of two sections–one for manufacturing and one for quality control. Both sections are automated and centrally controlled. The **manufacturing section** incorporates devices for cell washing, selection, electroporation, expansion, harvest, and formulation. Our approach, developed

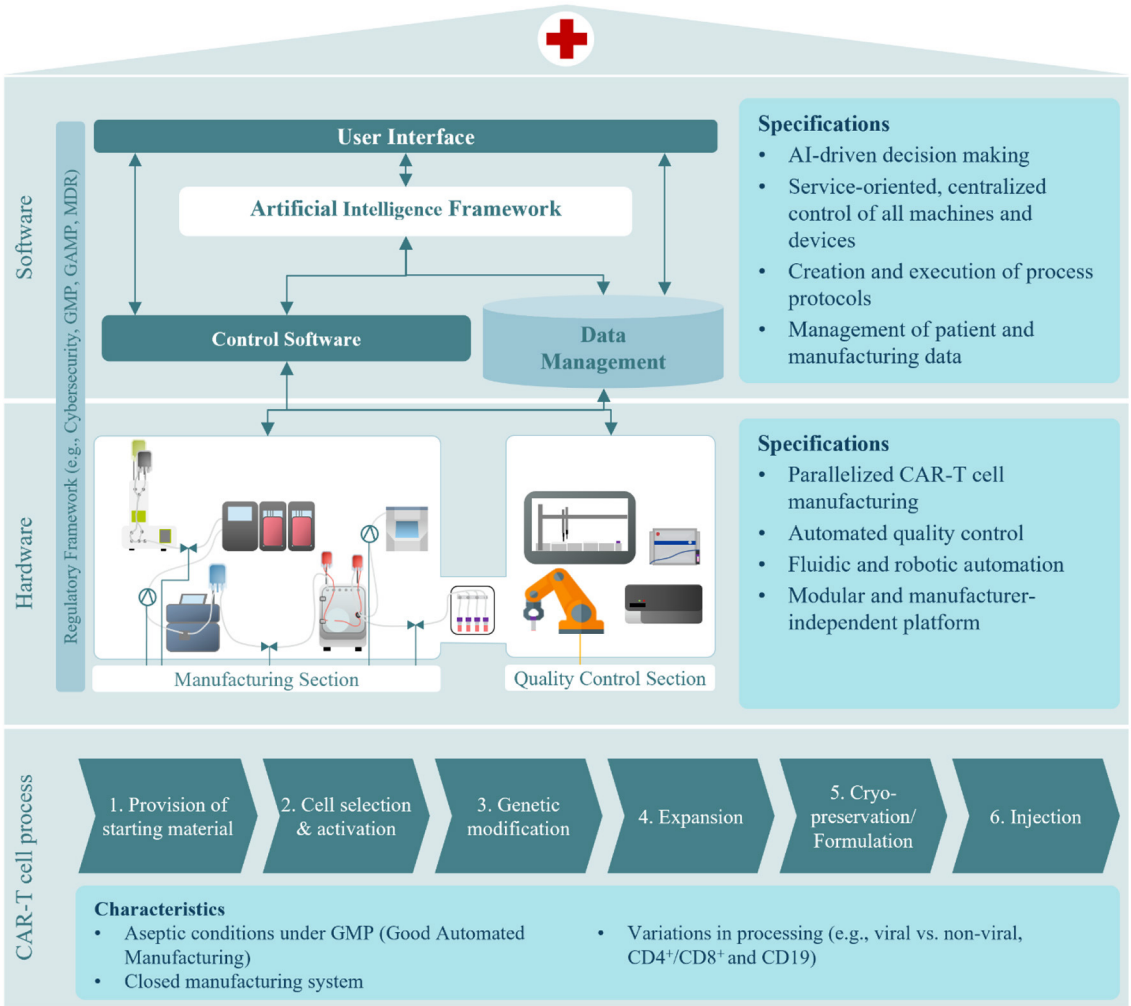

FIGURE 2 | Automated, AI-driven CAR-T cell manufacturing concept considering the CAR-T cell process, hardware, and software components.

in AIDPATH, realizes CAR-T cell manufacturing by automating tubing-kit-based devices and interconnecting the tubing kits with sterile connectors and tube welding. Cells are automatically transferred to their next process step by connecting tubing assemblies. This significantly reduces the need for a large number of highly trained personnel to manufacture CAR-T cell products while also greatly reducing the direct interaction of personnel and product and thus risk of contamination.

A key element of the manufacturing section is the integration of a sophisticated perfusion bioreactor, which not only enables the much-needed co-cultivation of $CD4^+$ and $CD8^+$ CAR-T cells but is also equipped with various sensors allowing the deployment of AI-supported control strategies, which will be developed during the AIDPATH project.

As manufacturing protocols integrate feedback loops based on the outcome of analytical measurements and product release is highly dependent on the time required for analytical assays, the **quality control section** tightly integrates quality control processes. Therefore, the platform is designed to pass cell samples aseptically and automated from manufacturing to quality control. The quality control section features devices to conduct analytics for cell quantity, viability, identity, and characterization of the subpopulations present. A liquid handler, flow cytometer, and cell counter are integrated using a six-axis industrial robot. The quality control section is completed by integrating automation enabling solutions for common laboratory tasks such as container capping and de-capping of material restocking.

CAR-T cell manufacturing is a cost-intensive process, not only because of its labor-intensiveness but also because of the resources required. Current semi-automated devices rely on a one-device-per-patient approach, allowing for parallelization only by increasing device numbers. This leads to a linear increase in investment costs for parallelized CAR-T cell manufacturing. To make autologous cell therapy manufacturing economically more attractive, **parallelized production** without a linear increase of costs needs to be implemented. In our concept, this is achieved by increasing the number of cartridges in the bioreactor

system. The longest step during manufacturing is expansion. Thus, parallelization can be achieved by exchanging each patient's incubation cartridge. As all manufacturing devices use closed tubing kits and these are replaced before the respective process step, the material of several patients may be processed in parallel without the risk of cross-contamination.

As CAR-T therapy is becoming a more established research area, the technologies applied for the generation of the therapy also evolve. Keeping up with technological advancements will require the integration of new devices. This is enabled by a **modular approach** to the manufacturing and quality control section. Integration of new devices for the manufacturing section is facilitated by the straightforward reconfiguration of the tubing assemblies, as these are equipped with standardized interfaces for sterile connection or welding. Retrofitting devices in the quality control section is also uncomplicated as the six-axis robotic handler features sufficient flexibility to provide different sample materials and carriers. Overall, this allows for **independence on manufacturers**, as various devices from different manufacturers can be integrated. Furthermore, although the platform is built for CAR-T cell manufacturing, the design is agnostic of cell type as the implemented technologies are also applied in other manufacturing processes. This makes the cell type manufactured on the platform in future developments flexible to a encompass variety of non-adherent, genetically engineered ATMPs.

For the **software** concept, two significant challenges arise. To enable end-to-end automation and eliminate all avoidable manual steps, a centralized execution, monitoring, and control of the entire process chain is required while integrating the various devices (22). Secondly, the AI models must be incorporated into the system to guarantee continuous data supply for model building and training and AI decision support for manufacturing control. Also, the software concept must consider the boundary conditions such as the modularity of the system, the volatile environment of ATMP manufacturing, and the regulatory landscape.

Currently, most devices in biotechnology are still being developed for manual operation. This makes automation difficult, as there are no interfaces for controlling the devices with external software or reading out data. Although standardized communication protocols such as OPC-UA and SiLA 2 are becoming increasingly important, they are not yet offered by most device manufacturers (23). Therefore, a middleware is needed for central control that generates a driver for each device. Each driver collects and sends data *via* a device's physical interface (e.g., USB, Ethernet) and then enables service-oriented communication with the **control software** *via* a standardized communication protocol. Here, the individual capabilities of the device (e.g., set temperature) are semantically described as a service. This semantically uniform description enables the flexible creation of protocols, integrating decisions (e.g., if the temperature is higher than X, then Y), and the low-effort integration of new devices (23, 24). A scheduling module ensures optimal machine utilization while scheduling all process steps including the parallelized manufacturing for the bioreactor cartridges. Digital batch records are generated automatically to avoid laborious manual documentation. In AIDPATH the software COPE is used and adapted to the requirements of the CAR-T cell process. The software was developed in several research projects for stem cell manufacturing (24, 25).

The making of AI models enables an intensive insight into biological processes and informed decision-making. However, they also require various patient data (e.g., age, gender, previous illnesses) and the manufacturing process (e.g., process and cell parameters, device information). These heterogeneous data sets are available in different qualities and formats and collected at different frequencies. Therefore, a **data management** framework must process all data using a standardized model, such as the OMOP Common Data Model (26), to ensure a general understanding and a straightforward analysis. Furthermore, data of different velocities must be integrated. Continuous data from sensors and devices in the manufacturing platform must be collected by a stream data platform (e.g., Apache Kafka) and made available to the AI models in aggregated form. Furthermore, a data storage platform is required that processes and stores batch data from patients and historical data sets. These data processing procedures and components form the foundation for the AI framework in which the various models are built, trained, and then deployed (27).

Another essential part of the automated AI-driven CAR-T cell manufacturing concept and the smart manufacturing hospital is the involvement of clinicians and technicians. Therefore, data can be integrated manually, automatically, and displayed in a user-specific way. Without expertise in software development, clinicians can create and customize process protocols using a drag-and-drop process creator. The decision support system, as part of the AI framework, transforms the results of the AI models into decisions and comprehensibly prepares them for human control and execution. This is brought together in a unified user interface, enabling centralized patient-specific process monitoring, data management, and manufacturing platform control.

From a regulatory point of view, our automated CAR-T cell manufacturing concept must comply with GMP and consider the GAMP guidelines (28). Also, the MDR [Medical Device Regulations (29)] will be taken into account. The smart manufacturing hospital's infrastructure will be designed to provide layers of in-depth cyber security and resilience to the manufacturing process. In case of a cyber incident, compromised segments are easily isolated to allow the infrastructure's continuous functioning.

ARTIFICIAL INTELLIGENCE IN CAR-T CELL MANUFACTURING

AI can gain crucial process insights into the cell's characteristics and behavior. This offers a great advantage for adaptive control of the whole process and the creation of personalized process protocols. Furthermore, AI can support economic platform operation in the smart manufacturing hospital by optimizing manufacturing schedules and resource management. Therefore, AIDPATH will develop different AI applications along the CAR-T cell manufacturing and therapy process.

To get deeper process insights on the CAR-T cell process and understand how patient-specific characteristics influence it, a **digital twin** will track the product through the entire manufacturing process and perform simulations on the cell behavior. Based on these insights the control software can adaptively control the bioreactor in the time-consuming cell expansion process. From the recorded process data of the bioreactor, such as oxygen or lactose, the cells' status can be determined and possible expansion strategies simulated (30–32). The process data is thereby supplemented by metabolomics data, due to their promising characteristics for quality control in personalized therapy (33, 34).

The planning of the therapy requires solving a complex **resource allocation problem** under substantial uncertainty and with frequent replanning. The complexity comes from varying production times and the number of resources needed, such as medical equipment or intensive care beds. Additionally, the time frame of therapy has to be adjusted on a patient-by-patient basis during therapy, depending on the progression. While conventional optimization algorithms reach here their limits, reinforcement learning is a promising method with the ability to cope with these challenges. Also, **adaptive scheduling** can integrate the manufacturing process on the platform optimally into the overall therapy process (35). In therapy planning, decision support for the physician facilitates central decisions. This enables a personalized therapy for each patient independent of predetermined values.

Since all these AI applications are used in a sensitive environment, one of the crucial aspects is **trustworthiness** (36). In the AI application domain, trustworthiness can be made tangible by asking two guiding questions: How well can one specify the application's behavior? What risks are introduced by the application, and how can they be dealt with? The specificity can be divided into the main pillars explainability, robustness, and security. Here, trust is increased by explainable results, robust predictions, and safe behavior of the application outside the actual work domain. Risks that continue to exist can be quantified and dealt with by risk management methods (37, 38).

DISCUSSION

This perspective has discussed an initial concept of how automated AI-driven CAR-T cell manufacturing can be implemented directly at the point of care in a smart manufacturing hospital. It focuses on the engineering perspective and how hardware and software components must be designed to manufacture autologous CAR-T cells efficiently. The regulatory framework is a significant obstacle that needs to be overcome before a wide-scale deployment is possible. While the facility design has been GMP-complaint and GAMP guidelines for the software have been considered, there is a need for precise regulatory guidance from EMA and the FDA on using AI-driven manufacturing platforms. This refers to a validation of a reliable functioning of the AI algorithms and the assurance of trustworthiness (e.g., appropriate data quality and quantity for training, possibility for continouos training) (39, 40). Another issue is economic considerations. Reduced manual, cost-intensive handling steps are set against automation costs. Comparing a similar system for automated stem cell production shows the potential for overall cost reduction (41). However, a health economic assessment for this concept will be the subject of future development in AIDPATH. In addition to purely economic considerations, the supply situation for patients must, of course, also be considered. Due to the parallelization of the bioreactor, high scalability and high throughput can be aimed. The resulting shorter production and delivery times positively affect the number of patient treatments. Nevertheless, a discussion is needed to what extent centralized and decentralized CAR-T cell production can coexist in the future. Another point that is still up for discussion is the operator model. Although the automated processes and an intuitive user interface allow operation by non-highly qualified personnel, it is still unclear to what extent such a system can be operated by hospitals or external service providers, such as pharmaceutical companies. In particular, it must be taken into account that the operation and maintenance of the hardware, AI and IT infrastructure will result in new tasks for the operator.

All in all, this is a promising concept that needs to be adapted and further developed to the rapidly changing market of cell and gene therapies in the coming years (42). Here, the focus must be on the transferability of the concept because CAR-T cell therapy is only the beginning of ATMP development and deployment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

The paper was written within the framework of the EU project AIDPATH (grant agreement number 101016909). All mentioned colleagues/companies are part of AIDPATH and therefore received funding from the EU within the scope of AIDPATH.

ACKNOWLEDGMENTS

The authors would like to acknowledge the contributions of their colleagues from Fraunhofer Institute for Cell Therapy and Immunology IZI, University College London, Foundation for Research and Technology (FORTH)-Hellas, SZTAKI, University Clinics Würzburg, Aglaris Cell SL, Sartorius Cell Genix GmbH, Fundació Clínic per a la Recerca Biomèdica, IRIS Technology Solutions, Red Alert Labs, Panaxea b.v., ORTEC b.v. This information reflects the consortium's view, but the consortium is not liable for any use that may be made of any of the information contained therein.

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Conflict of Interest: JJ was employed by ORTEC BV.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Companies Fraunhofer Institute for Cell Therapy and Immunology IZI, University College London, Foundation for Research and Technology (FORTH)-Hellas, SZTAKI, University Clinics Würzburg, Aglaris Cell SL, Sartorius Cell Genix GmbH, Fundació Clínic per a la Recerca Biomèdica, IRIS Technology Solutions, Red Alert Labs, Panaxea b.v., ORTEC b.v. were involved in the elaboration of the idea of the paper, the reviewing and the decision to submit the paper.

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