T-cell based advanced therapies, scientific hurdles and challenges for their development: the nonclinical testing strategies

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ABSTRACT — As the medical field evolves, so does the complexity of novel therapies. Diseases that were once a death sentence can now be treated with remarkable success. This is the case of several relapsed or refractory haematological cancers that can now be treated with CAR T-cells. This therapy takes advantage of the immunogenic power of T-cells and has transformed the field of personalised immunotherapy. Despite the challenges that this therapy faces, several CAR T-cell products have been approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA). This thesis aims to explore how the programs provided by both regulatory entities, as well as the flexibility demonstrated during the approval assessment, especially regarding the non-clinical development, have influenced the path followed by these advanced therapies, using MAXQDA software to facilitate the analysis. Firstly, a comparison between the approval journey of the EMA and the FDA was provided, followed by an analysis of the non-clinical requirements to which CAR T-cells should comply, being both cell-based and gene therapy medicinal products (GTMPs). Finally, a case study like exercise was performed to compare the assessment reports of two CAR T-cell-based products, two other GTMPs and one COVID-19 vaccine, all using viral vectors to induce the desired genetic modification, showing the value of using a risk-based approach during the design of non-clinical studies. Results also allowed a discussion regarding the common goals of non-clinical studies before the start of clinical trials and the use of relevant animal models.

KEYWORDS: Lymphocytes**,** CAR T-cells, Marketing Authorisation Application, Biologics License Application, Advanced Medicinal Products, Risk-Based Approach, Gene Therapy

1. INTRODUCTION

The medical field is continuously evolving, as new medicines and therapies are constantly emerging. This was especially evident during the coronavirus disease (COVID)-19 pandemic and the short time-range at which the COVID-19 vaccines started to be administered to the global population.

However, medicines today are much more than vaccines and pills, and, particularly in the last few years, there has been a change towards patient-specific therapies. These enable the treatment of diseases and conditions that used to have very limited treatment options and expected outcomes. However, the pharmaceutical industry is also moved by profit, hence the development and production of a specific product for each patient at an accessible price can be challenging. Notably, regulatory entities such as the European Medicines Agency (EMA) and the United States Food and Drug Administration

(FDA) have been adapting their protocols and directives to ensure that personalised therapies reach the patients as soon as possible, without compromising its safety and efficacy.

Thus far, one of the main targets of personalised medicine has been cancer therapy, which makes sense since cancer is intrinsically related to the immune system and even the genome of each patient[1].

Cancer therapy can be divided into non-immunotherapies, such as chemotherapy, radiotherapy and tumour removal surgery, and immunotherapies, which are treatments that directly modulate the patient's own immune system to achieve beneficial clinical outcomes[2]. As cancer continues to be one of the leading causes of death in the developed world[3], and because there is still a lack of effective cancer treatments (especially after remission) in some types of cancer, as well as a high variability of outcomes, it is logical that there is intense research in the field of cancer immunotherapy.

Some of the existent targeted immunotherapies take advantage of the patient's specific subset of immune cells – the T lymphocytes – to fight against cancer cells. The use of gene transfer technology enabled the engineering of T-cells to express chimeric antigen receptors (CARs), which are capable of specifically recognising their target antigen in cancer cells[4].

The first approval of a CAR T-cell therapy by the FDA and the EMA, in 2017 and 2018, respectively, represented a big step for the use of immunotherapy to treat cancer, particularly haematological cancers. This opened a new door and since then similar therapies have been approved and are currently being developed[5].

CAR T-cells belong to the category of advanced therapy medicinal products (ATMPs) in Europe and Cellular & Gene Therapy Products in the United States, which are required to follow multiple regulatory guidelines, in particularly, regarding the nonclinical development. This development is fundamental to allow a better risk monitoring during clinical trials and to obtain supportive data for the approval of medicinal products.

This thesis was divided in three major topics, including the characterisation of CAR T-cells, drug development and nonclinical studies design. After an extensive literature review, several comparative studies were conducted, with the assistance of MAXQDA software.

2. BACKGROUND

T-cells and the potential of T-cell based therapies

The immune system, which is a vital part of human survival, comprises the innate immune system and the adaptive immune system. Innate immune cells recognise general danger patterns and, on the other hand, the adaptive immune system cells, T and B lymphocytes, have unique receptors called T-cell receptors (TCRs) and B-cell receptors (BCRs), respectively, that recognise specific antigens. B lymphocytes have the role of presenting the antigens to T lymphocytes and producing antibodies that neutralise the pathogen. T lymphocytes can be divided in CD8+ T-cells, which are usually cytotoxic, or CD4+ T-cells, which can be helper or regulatory[6], [7].

During an immune response, tissue-resident antigen presenting cells (APCs), such as dendritic cells and macrophages, are activated to take up cellular and pathogen debris and then migrate into the T-cell zones of the local secondary lymphoid organ (SLO). During this migration, APCs process and present pathogen-derived antigens in the context of class I and class II of the major histocompatibility complex (MHC) and also release cytokines depending on the type of pathogen they have encountered. The naïve T lymphocytes in the SLO, on engagement of their TCR and depending on the surrounding cytokines are genetically programmed into the appropriate T-cell subset, and then migrate to the problematic site. Upon differentiation, T-cells also start producing cytokines that feedback the process, which amplifies and balances the immune response. After the problem is resolved, the T-cells die off[6].

Ultimately, T-cells have the power of eliminating outside and inside threats to the organism with a precise and effective mechanism while also regulating their own response, making them an attractive focal point for immunotherapy.

Engineering T Lymphocytes for cancer

Adoptive cell therapy (ACT) is a type of cancer treatment where immunocompetent cells are collected from patients, reactivated, enhanced and/or expanded, and then transferred back into the patients. Examples of ACT include tumour-infiltrating lymphocytes (TILs), TCR T-cells, and CAR T-cells therapies[8]. The present work focuses on CAR T-cells, as these are the main component of the recently approved medicinal products.

CARs are genetically engineered receptors that mimic TCR activation, redirecting specificity and effector function toward a specific antigen. They

have a modular structure with four domains: an antigen-binding domain, a hinge, a transmembrane domain, and an intracellular signalling domain. It is called chimeric, because the structure of the receptor is a fusion of the antigen recognition portion of an antibody (in cancer therapy, this is specific for a tumour cell surface antigen) with the intracellular signalling domain of a TCR plus additional intracellular costimulatory molecules that help activate the immune attack[9].

Building the four modular components of CARs is arguably the most critical step in any CAR T-cell therapy. Enhanced engineering strategies are and will be able to improve the safety and efficacy of CAR T-cell therapies, broaden the range of cancers responsive to such treatments and facilitate more rapid, reliable, and efficient production of these products.

CAR T-Cell Therapy in the Clinic

CAR T-cells need to be administered in qualified treatment centres due to the complexity of the procedure. First, blood is drawn from the patient and T lymphocytes are separated out through the process of leukapheresis. The T-cells are then purified, reprogrammed into CAR T-cells ex vivo (through the transduction of the CAR), expanded, and then frozen for future administration. Before the reintroduction of the CAR T-cells, the patient undergoes conditioning chemotherapy (i.e., lymphodepleting chemotherapy) to promote engraftment and proliferation of transferred cells. Following tumour burden reassessment, the cells are thawed and infused[10], as seen in figure 1.

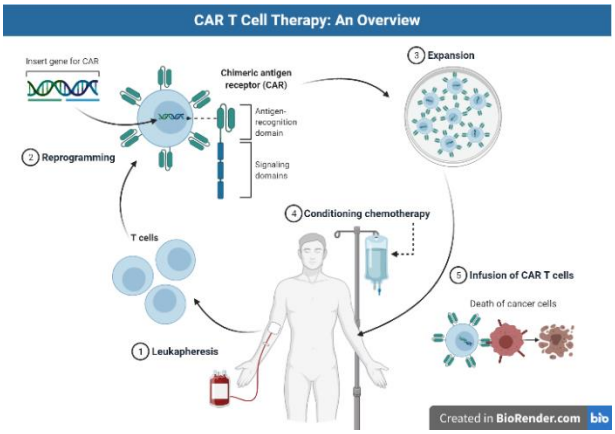

Figure 1 – Schematic representation of the process followed for CAR T-cell administration

B-cell malignancies have been an attractive target for CAR T-cell therapies because they express B cell lineage-specific molecules such as CD19 and CD20 that are not expressed in other tissues. The types of refractory or relapsed (r/r) haematologic cancers that have showed notable antitumour effects following CAR T-cell treatment include acute lymphocytic leukaemia (ALL) and Non-Hodgkin lymphoma[11].

Limitations and Challenges of CAR T-cell therapy

Despite the impressive outcomes shown in the field of CAR-T-cells, it is still currently facing major challenges, namely, antigen loss (which can occur via antigen escape – patients relapse with a phenotypically similar disease that lacks surface expression of a CD19 molecule – or lineage switch – patients relapse with a genetically related but phenotypically different malignancy) and suboptimal persistence *in vivo*; obstacles regarding solid tumours; CAR T induced toxicities and other side effects; manufacturing challenges; and burden on health care systems.

Currently, the main obstacles in solid tumour CAR-T therapy are the inefficient T-cell trafficking, suboptimal antigen recognition specificity, and immunosuppressive tumour microenvironment – physical barriers and T-cell inhibitory signals[12].

The activation and rapid expansion of CAR T-cells once these are infused into the patients is associated with high systemic levels of cytokines, which reflects the strong interactions of CAR T-cells with cancer cells and/or cells of the host's immune system. In some patients, cytokines can reach toxic levels. These toxicities include cytokine-release syndrome (CRS) and neurotoxicity, also referred to as immune effector cell-associated neurotoxicity syndrome (ICANS) or CAR-related encephalopathy syndrome[9].

Another main challenge of this largely personalised medicine is the development of efficient technologies and cost-effective clinical manufacturing platforms to support its commercialisation[13]. To stimulate the use of these innovative medicines, early evaluation and shaping of the price and reimbursement options are desired from the pre-clinical stage, and manufacturers must play a role in facilitating implementation and relieving the administrative burden on healthcare systems.

Allogeneic CAR T-cell therapy

The development of universal off-the-shelf CAR T-cells (using allogeneic donor T-cells) readily available for patient treatment, potentially at reduced cost, would significantly increase access to this class of therapeutics. Allogeneic CAR T-cell therapy allows a broader access, overcoming the manufacturing difficulties of producing CAR T-cells

for each individual patient, providing a more functional, potent product for malignancies where T-cell dysfunction is common and cannot be fully reversed during the manufacturing process[14], [15], and potentially reducing the burden on healthcare systems.

The approval journey of medicinal products

The complete process of drug development goes from early drug discovery with basic biological research, disease modelling, and target discovery, to preclinical studies with in vitro, ex vivo, and in vivo models, and clinical development with human subjects. Finally, the medicine is submitted to regulatory review and approval, after which it goes through post-market monitoring and pharmacovigilance.

Upon research and discovery, a promising group of therapies emerges and progresses to the non-clinical development, which includes pharmacology, pharmacokinetics, and toxicology. Pharmacology relates to the organism's biological response to the medicinal product and evaluates its efficacy while demonstrating the "proof of concept". Pharmacokinetics relates to the distribution of the medicinal product throughout the organism. And, finally, toxicology studies are used to evaluate any side effects that the medicinal product may have on the organism[16].

After this, a few selected medicinal products progress to the clinical development, which include phase I, phase II and phase III clinical trials. These types of clinical studies have an increasing number of participants and durability, providing an increasing amount of data[17].

After the medicinal product is approved, it enters the pharmacovigilance stage, where it is subjected to post-marketing monitoring, and it can enter phase IV clinical trials. Usually, the applicant is required to submit periodic safety updates to the regulators[18].

Regulatory Framework in the US and the EU

Both the FDA in the US and the EMA in the EU offer numerous guidelines for the quality, non-clinical and clinical development of all types of medicinal products, and both provide scientific advice and various programs to assist the development and approval of medicinal products. Notably, even though the EU consists of 27 different member states, most complex and innovative medicines go through the centralised authorisation procedure, in which the authorisation is given by the European Commission (EC) based on EMA's recommendation[19].

The applicant can submit the Marketing Authorisation Application (MAA) in the EU and the Biologics License Application (BLA) in the US to get regulatory approval. Both the EMA and the FDA have orphan designation programs that offer a range of incentives to the developers of products for rare diseases. In both the US and the EU, the applicant may also apply for expedited development programs, which are usually tailored to medicinal products that treat serious conditions and/or address an unmet medical need. The FDA established the Fast-Track Designation, the Breakthrough Therapy Designation, and the Regenerative Medicine Advanced Therapy Designation[20]–[22], which all make the medicinal products eligible to Priority Review (i.e., the review of the BLA is shortened from the standard 10 months to 6 months)[23] and Rolling Review (i.e. data is evaluated as it becomes available). The Breakthrough Therapy Designation is equivalent to the EMA's PRIority MEdicines (PRIME) Scheme[24], which was designed for medicines that offer a major therapeutic advantage over existing treatments or target unmet medical needs, giving the applicant access to a dedicated contact point, additional meetings and other regulatory support, as well as access to Accelerated Assessment, which reduces the review of the MAA from the standard 210 days to 150 days[25].

Additionally, in the US, ATMPs are referred to as Cellular and Gene Therapy products and the responsible regulatory offices are the Centre for Biologics Evaluation and Research (CBER) and the Office of Tissues and Advanced Therapies (OTAT). For the EMA, the Committee for Advanced Therapies (CAT) is responsible for submitting a draft opinion to the Committee for Medicinal Products for Human Use (CHMP), which then delivers the final opinion to the EC.[25]

Risk-based approach for ATMPs

The regulators in pharmaceutical industry (both the EMA and the FDA) started to adopt a new methodology called risk-based approach with the purpose of having an efficient development and manufacturing without compromising the quality and safety of medicinal products. In Europe, this methodology has been of particular relevance for advanced and innovative therapies, which resulted in the introduction of the EMA's Directive 2009/120/EC in late 2009[26]. The methodology of risk-based approach is centred on the identification of risks and associated risk factors of an ATMP and the establishment of a specific profile for each risk. Risk is defined as an adverse effect resulting from the clinical use of the ATMP and that is of concern to the patient and third parties (such as caregivers and offspring). Risk factor is defined as a "qualitative

or quantitative characteristic that contributes to a specific risk following handling and/or administration of an ATMP", which is usually related to the nature of the ATMP, non-cellular components, biodistribution, manufacturing issues and clinical aspects[27]. This profiling reduces unexpected occurrences as it helps prevent every possible outcome during clinical administration. By inducing regulatory flexibility and revisions as more knowledge is gained, the risk-based approach is established on the basis of prevention and leads to an increased benefit-risk ratio to patients.

3. METHODOLOGY

Research Questions and Data Research

This thesis involved different types of studies, conducted using diverse information sources. The different research components addressed were: perform an overview on the currently approved T-cell based medicinal products; understand the approval journey of a new medicinal product reaching the market; identify the existing regulatory guidance, from the EMA and from the FDA, and assess the applicability of these guidelines for meaningful non-clinical development programs, identifying the challenges encountered during non-clinical development for CAR T-cell products and other similar medicinal products. For this purpose, an extensive literature review was conducted using the b-on library[28] in which some keywords and expressions used included: T-cell function, CAR T-cell Therapy, Engineered T-cells, Immunotherapy.

Research questions included: 1)"What are the approved CAR T-cells?"; 2)"What are the non-clinical studies expected for CAR T-cells?"; 3)"How can a risk-based approach influence the non-clinical data package?"; 4)"What risks can be identified during the analysis of multiple assessment reports?"; 5)"What do all non-clinical developments have in common?".

The approved CAR T-cells therapies in each geographic region were listed and addressed. In the FDA's website, it was easy to access this information following Vaccines, Blood, and Biologics > Cellular & Gene Therapy Products > Approved Cellular and Gene Therapy Products, However, in the EMA's website, it is not possible to conduct a search within a specific pharmaceutical group and so a literature review was performed to attain the CAR T-cell products approved in the EU at the time of analysis.

Analysis of Regulatory Documents

Due to the extent of the documents considered for analysis, the software MAXQDA was used. MAXQDA is a software program designed for computer-assisted qualitative and mixed methods data that allows data storage, classification, and management, allowing, for example, the assessment of data through comparison diagrams[29]. The EMA website was a valuable source of information for discovering the several guidelines and documents applicable to ATMPs, namely following the path: Human Regulatory > Research and Development > Advanced Therapies > Scientific Guidelines, as well as enabling a better understanding of the regulatory process of ATMPs in Human Regulatory > Overview > Advanced Therapies. The FDA's website was also consulted following Vaccines, Blood, and Biologics > Cellular & Gene Therapy Products > Cellular & Gene Therapy Guidances to find the most pertinent Guideline for the non-clinical studies of CAR T-cell-based therapies, namely *Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products*[30]. This document was used for comparison against the EMA's *Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells*[31].

Analysis of the case studies

After gaining a broad view of non-clinical development design, the approved CAR T-cell therapies' European Public Assessment Reports (EPARs) were compared to two other approved GTMPs and to the recently approved COVID-19 vaccine Astrazeneca. All the EPARs were retrieved from the EMA's website by searching for each product's name. This analysis was also facilitated by the software MAXQDA.

4. RESULTS

Comparative supportive programs and timelines for accelerating development and approval of CAR T-cells in the EU and US

Regarding the type of programs and designations that were associated with the development and approval of CAR T-cell therapies in the EU, all the approved medicinal products received Orphan and PRIME designation; Tecartus received a CMA and both Yescarta and Kymriah received a standard marketing approval[32]–[34]. In the US, Tecartus, Yescarta and Kymriah received an Orphan Designation, and were granted Priority Review and Breakthrough Therapy designations; Tecartus and Yescarta were approved under the Accelerated Approval program, and Kymriah received a standard marketing authorisation. Breyanzi, which is only approved in the US, received an Orphan

Designation, and was granted Breakthrough and Regenerative Medicine Advanced Therapy Designation designations, receiving a standard marketing authorisation[35].

Besides all the pre-approval programs, both the EMA and the FDA stimulate post marketing authorization tools for these medicinal products, such as educational programmes for patients and healthcare professionals and the submission of periodic safety update reports and post-authorisation safety studies[33], [35].

The innovative therapies that target rare conditions or diseases take the biggest advantage of the programs mentioned in this chapter. Not only their applicants receive fee reduction and extra scientific support, but the development and assessment paths are expedited, without compromising the efficacy and the safety of these products and allowing them to reach the patients sooner. The post-marketing programs allow the regulatory agencies to make sure the medicinal products are being administered in as intended and confirm their health benefits.

Details of Marketing Approval of CAR T-cell-based therapies

All the approved CAR T-cell therapies are ATMPs containing autologous T-cells genetically modified *ex vivo* by viral transduction to express a CAR comprising a murine anti-CD19 scFv linked to CD28 or 4-1BB co-stimulatory domains and CD3-zeta signalling domain[32]–[35] and are summarised in table 1.

Table 1 – Summary of the approved CAR T-cell therapies

	Kymriah	Yescarta	Tecartus	Breyanzi
Construct	Anti-CD19-4-1BB-CD3ζ	Anti-CD19-CD28-CD3ζ	Anti-CD19-CD28-CD3ζ	Anti-CD19-4-1BB-CD3ζ
Manufacturer	Novartis Pharmaceuticals Corporation	Kite Pharma, Inc.	Kite Pharma, Inc.	Juno Therapeutics, Inc., a Bristol-Myers Squibb Company
Indication	1. Paediatric and young adult patients up to 25 years of age with r/r B-cell ALL 2. Adult patients with r/r DLBCL after two or more lines of systemic therapy	Adult patients with r/r large B-cell lymphoma (DLBCL and PMBCL)	Mantle cell lymphoma	Adult patients with r/r large B-cell lymphoma, after two or more lines of systemic therapy
FDA's approval	Indication 1 – 30 August 2017 Indication 2 – 1 May 2018	18 October 2017	24 July 2020	5 February 2021
EMA's approval (CHMP positive opinion)	Indication 1 and 2 – 28 June 2018	28 June 2018	15 October 2020	-
Authorised in EU	23 August 2018	27 August 2018	15 December 2020	-
Cost (US$)	475,000	373,000	373,000	410,300

One aspect which appears common for the first approved therapies in EU and US is the earlier timelines for the marketing authorization observed in the US, especially for Kymriah and Yescarta, which were approved in the US around a year before being approved in Europe. This may deserve further reflection on the reasons behind.

Non-clinical Studies supporting CAR T-cells development and approval

Before analysing the supportive non-clinical studies for the approved CAR T-cell therapies, a comparison of EU and US guideline requirements has been performed to allow a better use of available documentation.

Because both the EMA and the FDA are two different regulatory entities, it is interesting to understand whether they have the same non-clinical requirements for CAR T-cells and other medicinal products with genetically modified cells. For this study, an FDA document aimed for providing guidance for the development of preclinical studies of cellular and gene therapy products (FDA's equivalent to ATMPs), called FDA's *Guidance Document for Preclinical Assessment of Investigational Cellular and Gene Therapy Products*[30] was uploaded into the MAXQDA software and compared with the most recent *Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells*[31] from the EMA. This analysis suggested that both regulators have very similar recommendations for this type of medicinal product, which complies with the idea that despite some differences in the regulatory process, the EMA and the FDA tend to follow the same reasoning, and often work together to uniformise the regulatory pathway for new medicines. In fact, the divergent non-clinical studies presented above may be due to the way the guidelines are written, and not necessarily to divergent information. For this reason, and because the search of documentation regarding non-clinical studies is easier to perform on the EMA's website, the following studies were conducted based on the documents and information from the EMA, as the extraction of data is facilitated comparing to the documents provided by the FDA.

Cell-based products and GTMP non-clinical requirements comparison and relevance for CAR T-cells

This chapter allowed to understand the differences and similarities of the non-clinical studies required for each class of ATMPs – gene therapy and cell based medicinal products, keeping in mind that the *Guideline on human cell-based medicinal products* was available before the *Guideline on the risk-based approach according to Annex I, part IV of Directive 2001/83/EC applied to Advanced Therapy Medicinal Products* was available. For a more effective analysis, both guidelines were uploaded into the MAXQDA software.

Even though GTMP and cell-based products both belong to the category of ATMPs, there were noticeable differences in the recommendations for each study mentioned in this chapter. Particularly, non-clinical studies for genetically modified products tend to focus more on the effects of the transgene in its intended target as well as in other relevant parts of the organism. For cell-based products, cell mobility and differentiation are essential factors to address during these studies. However, the goal of each study is the same for both types of products.

The similarity of adjusting the non-clinical requirements to each product, covered in both guidelines, suggested that even when the risk-based approach was not recognised by the EMA, it was already considered by the regulators to a certain extent. It was concluded that the extent of data to be included in the MAA is evaluated on a case-by-case basis and depends on the perceived risks related to the product, previous scientific knowledge, and clinical experience with similar type of products.

Case studies analysis

Since the search of documentation regarding non-clinical studies is easier to perform on the EMA's website, the studies conducted to compare the available CAR T products were based on the documents and information from the EMA.

Bearing in mind this thesis's goal and methodology and due to the complexity of CAR T-cell therapy and the fact that it falls into two different ATMP category, two other approved GTMPs – Strimvelis and Zolgensma – were analysed for comparison purposes. Furthermore, since the COVID-19 pandemic has erupted during the research for this work and it was such a disruptive event intrinsically connected with the pharmaceutical industry, the assessment report of the first viral vector-based COVID-19 vaccine approved in the EU – Astrazeneca – was included in this analysis. This comparison was acceptable since all medicinal products are manufactured using the same technology of viral vectors, which was explored in this chapter. The medicinal products used for this case study like exercise and the respective viral vector characteristics are summarised in table 2.

The influence of a Risk-based approach on non-clinical studies

From the analysis conducted, it was evident that the extent of non-clinical studies provided depends not only on the type of product but also on previously existing knowledge, since, for some of the subtypes of non-clinical development, the applicants provided a previously conducted study to either fully support the information needed or as a complement to studies conducted by the applicant.

Table 2 – Selected case studies and respective viral vector characteristics

	Kymriah	Yescarta	Astrazeneca	Strimvelis	Zolgensma
Type of vector	Lentiviral	Retroviral	Adenovirus	Retroviral	AAV
Replication	Defective	Incompetent	Deficient	Deficient	Deficient
Integration (*)	Integrating	Integrating	Non-integrating	Integrating	Non-integrating
Encoding	CAR construct	CAR construct	SARS-CoV-2 spike protein	Human ADA sequence	Human SMN1 gene
Gene therapy strategy	*Ex vivo*	*Ex vivo*	*In vivo*	*Ex vivo*	*In vivo*

AAV: adeno-associated vector, ADA: adenosine deaminase, SMN: survival motor neuron

Furthermore, even if the risk-based approach was not mentioned directly, it was possible to see some evidence of its use in the non-clinical data required. This methodology allowed the applicants to focus more on pertinent studies and to omit others, leading to a more meaningful non-clinical development. This regulatory flexibility is continuously evolving as more data are generated.

To better discuss the risks and risk factors of the medicinal products, they were divided based on the type of vector used and where the genetic modification takes place – *in vivo* or *ex vivo*, in order to enable the identification of risks and risk factors.

Integrating vector and ex vivo genetic modification

Genetically modified cells are collected from the patient, engineered *ex vivo*, expanded and then administered back to the patient through an intravenous infusion. As expected, this process has associated risks, which were approached during the assessment reports of these products, and that include treatment failure, toxicity safety issues, and tumour formation.

The possibility of treatment failure is a risk for every medicinal product. However, both CAR T-cells and Strimvelis were developed for unmet medical needs, which means that if the therapy fails, it is not likely that the patient has other treatment options, hence it is essential to study the efficacy of these treatments. However, the fact that these are personalised immunotherapies means that there is a lack of relevant animal models. Therefore, the applicants are highly dependent on *in vitro* models to study the efficacy before the first clinical trials. In the case of these therapies, the long-term efficacy should also be demonstrated to confirm the expected long-term effects. For this reason, the applicants of Yescarta and Kymriah focused on the

specificity, persistence and expansion of the CAR T-cells. In the case of Strimvelis, the engraftment and differentiation capacity of the CD34+ cells were investigated to ensure that the therapeutic desired outcome is reached.

Due to the expected strong immunological effect of the CAR T-cells against the CD19-presenting cancer cells, some adverse effects might occur, either by excessive cytokine production or due to unwanted targeting of cells/organs. Therefore, the applicants of Yescarta and Kymriah focused on measuring cytokine production and evaluating on-target/off-tumour toxicity. However, the limitations of animal models are an obstacle to perform these types of studies. In the case of Strimvelis, its intended purpose is to restore the production of the adenosine deaminase (ADA) enzyme at or below physiological levels, hence toxicity studies related to the effects of the enzyme are not relevant. However, this medicinal product presents a risk of autoimmunity caused by the presence of anti-ADA antibodies, which could not be addressed during non-clinical development.

When it comes to therapies that use integrating vectors to alter genetic material, one concern that should be addressed is insertional mutagenesis. This mutagenic event can alter the patient's gene transcription, posing a risk of cell transformation and eventually tumour formation. Therefore, it is important to perform insertional mutagenesis analysis or other type of analysis that assesses the integration site in case integration occurs. In the case of Strimvelis, which contains haemopoietic stem cells, the stem cell proliferation capacity may also lead to tumorigenic events and is therefore important to study this characteristic before testing the medicinal product in humans. The fact that Strimelis' applicant struggled to assess the risk of clonal expansion and tumour arising from the genetically modified cells was due to the limitations of animal models. Hence, the applicant agreed to a 15 year follow up of patients in clinical practise (which is the same follow-up timeframe agreed for CAR T-cell therapies) and monitoring of potential mutagenicity.

Non-integrating vector and in vivo genetic modification

Both Zolgensma and Astrazeneca are based on the administration of non-integrating vectors into the patient and subsequent protein production. Both have received a conditional marketing approval by the EMA, which means that the applicants are required to submit additional data. Even though the therapeutic intent is different, the stages following administration are comparable: the vector containing the transgene is administered, the cells are transduced, the cells start producing the protein encoded by the transgene. The vectors used in these medicinal products are non-integrating, which means the new genetic information does not integrate into the human genome and hence cannot alter the cells and lead to tumorigenicity. Therefore, this is not a focal point for either Zolgensma or Astrazeneca.

Even though some crossing of collected information between the two medicinal products was expected due to the similarity of the vectors and the fact that the transgene expression only occurs in vivo, the non-clinical studies addressed in the assessment reports were quite different.

From the analysis of the assessment reports it was noticeable that while Zolgensma's non-clinical studies focused on the distribution of the vector and transgene expression, Astrazeneca's focused on assessing the immunogenicity and subsequent protection rather than focusing on the vector and the transgene. For these reasons, the identified risks of each medicinal product and the chosen non-clinical development approach were different.

The administration of viral vectors may induce an immune response. Immunogenicity is not desired in the case of Zolgensma, but it is an expected effect upon administration of the COVID-19 vaccine. For Zolgensma, it was considered essential to assess the target-specificity, during pharmacokinetic studies, by measuring vector transduction and transgene expression to confirm a CNS biodistribution of the vector as well as efficient transgene expression, as the production of survival motor neuron (SMN) protein by motor neurons is considered essential to the success of the therapy. However, the vector and the transgene do not distribute solemnly to the motor neurons. Hence, it was indispensable to evaluate the immune cell response directed against transduced cells and the AAV capsid, as well as assessing the measurement of antibody titers against the vector and the transgene, even more so after evidence of possible cardiovascular, liver and dorsal root ganglia toxicity during non-clinical studies. In fact, liver toxicity was approached during multiple non-clinical studies, as this was the organ where most SMN1 copies were found, and where the persistence of high vector DNA and protein overexpression occurred. The immune response upon AAV-mediated *in vivo* transduction can lead to unwanted immunogenicity and cause serious complications to the patient, hence this is a risk of these therapy and was flagged to be closely monitored in clinical trials.

Notably, even though Astrazeneca goal is to produce an immune response that will confer

protection against SARS-CoV-2, the immunogenicity should be against the S protein on the surface of the transduced cells and not against the vector. The applicant conducted several immunogenicity studies that focused on the immune response against the spike protein regarding the stimulation of neutralising antibody and cellular immune responses. However, data on antibody subtypes, Th1/2-biased response, T-cell subtyping and determinations of neutralising antibodies after vaccination and challenge was either limited or completely absent. Furthermore, the applicant conducted vector biodistribution studies using a similar virus and using the same platform with a different insert, but the methods used were not validated, and the ongoing biodistribution study still has not been submitted. The applicant did not assess protein distribution nor validly assessed the stimulation of antibodies, such as autoantibodies, which might have had contributed to the identification of possible risk factors causing unwanted immunogenicity. It is important to recall that, after the approval of this vaccine, and after million vaccine doses had been administered, several cases of unusual immune thrombotic events in combination with thrombocytopenia were observed in patients after vaccination. The vaccine-induced thrombotic thrombocytopenia disorder (which involves the production of an autoantibody) was observed in patients receiving this vaccine[36]. The pathophysiological mechanism has not been established, and it is still not possible to identify specific risk factors, however, the EMA determined that a possible explanation to the combination of blood clots and low blood platelets is an immune response.

The fact that the viral vector is administered directly into the patient means that there is a higher risk of it transducing germline cells. For this reason, both applicants provided some type of reproductive toxicity study.

The limitations of animal models and their impact on a risk-based approach

It was concluded from the previous analysis of assessment reports that non-clinical studies should be performed in relevant *in vitro* and animal models according to the target cell population, clinical indication and route of administration. Arguably, the most important factor when considering the animal model should be its ability to generate robust and predictive data, as to comply with the 3Rs (reduction, replacement, refinement) principles[37].

All the therapies using genetically modified cells used only one animal model – mice – which is widely employed in drug development. The applicants of these medicinal products chose to provide comprehensive *in vitro* studies and carefully discuss their limitations, since efficacy and safety data obtained in animal models can be challenging to extrapolate to humans.

Ultimately, there is no single optimal preclinical model for CAR T therapy, but developments in breeding transgenic mouse strains, improvements in the humanization of murine immune systems, and the combination of multiple animal models will provide more information of different aspects regarding CAR T-cells. Moreover, the regulatory agencies encourage applicants to replace animal testing with *in vitro* or *ex vivo* studies, with the use of cell- and tissue-based models, organoids and microfluidics, in silico models or other non-animal approaches, when appropriate and applicable. One particularly interesting microfluidics-based methodology is the “organ-on-a-chip”, which aims to mimic the “key organotypic cellular architecture and functionality, 3D extracellular matrix, biochemical factors, and biophysical cues” in a more compact and smaller manner, with the purpose of disease modelling and drug screening. For blood diseases, such as blood cancers, an even more predictive model would be the “body-on-a-chip” since blood circulates throughout the entire body. This model mirrors the physiology of the entire human body using a “single platform for drug pharmacokinetic and pharmacodynamic analyses”, holding great promise for advancing the therapeutic screening of cancer immunotherapies[38]. Besides suggesting the use of different types of non-clinical models, the EMA also proposes that, when feasible, several non-clinical aspects can be addressed in one study.

On the other hand, both Zolgensma and Astrazeneca used multiple animal models, including animals with body size and anatomy closer to those of humans, such as non-human primates and pigs. This is because studies in larger animal models were considered relevant for these therapies.

5. CONCLUSIONS

The approval of CAR T-cell therapies has revolutionised the field of cancer immunotherapy. They take advantage of the power of T lymphocytes to eliminate cancer cells and treat patients that would otherwise not have other treatment options. Today, CAR T-cell therapy research continues, not just using CD19, but targeting other antigens, and not only for haematological cancers but for the treatment of solid tumours and even other types of diseases. Their efficacy and safety are only expected to increase with the development of fourth

generation CARs and the increased knowledge gained from clinical experience.

CAR T-cells fall in the scope of advanced therapies, which are more complex than conventional drugs and so they require an adapted development. Notably, the more knowledge and clinical experience there is regarding a medicinal product, the more accurate its associated risks and risk factors are defined. The identification of these risks can and should influence the type of studies conducted during non-clinical trials, and should help simplify the non-clinical development, even for advanced therapies. This justifies the introduction of a risk-based approach when considering the non-clinical data package that should be conducted prior to clinical trials and that is submitted for the approval of an ATMP. In fact, the risk-based approach can contribute to a more focused and expedited non-clinical development, allowing the first clinical trials to start sooner and therefore also reducing the investment of the companies.

The CAR T-cell therapies' non-clinical package was then compared to other GTMPs and also to the COVID-19 vaccine Astrazeneca. Even though gene therapy products do not include vaccines against infectious diseases, the Astrazenca vaccine is not a "conventional" vaccine, as it depends on a genetic modification for cells to start producing the spike protein and only then the immune response against the protein is expected. As this genetic modification also involves the use of viral vectors, the comparison was considered appropriate. It was expected, however, that the non-clinical studies conducted for the vaccine were approximate to the ones conducted for the advanced therapies, as it also consists of the delivery of a vector to induce a genetic modification and subsequent protein production. It could be interesting to try to understand why this type of vaccine is not considered an advanced therapy and why the non-clinical studies performed did not investigate the complications associated with gene therapies. For example, the risks encountered for Zolgensma related to liver and cardiac toxicity caused by the persistence of high doses of vector DNA and overexpression of the protein were not addressed for the vaccine. And if both Astrazeneca and Zolgensma are non-integrating vectors being administered into the person's body, shouldn't the associated risks be similar? This similarity was not reflected during the analysis of the non-clinical development programs and the rationale behind this development appeared to be different from the rationale used for the advanced therapies. In fact, this viral vaccine is not considered a GTMP, but it used to belong to the same category of these products (i.e., gene transfer medicinal products). The analysis seemed to indicate that a risk-based approach might not have been considered for the Astrazeneca vaccine, and this should be further investigated.

The medicinal products mentioned in this work have all allowed to fill unmet medical needs and contribute to the improvement of patient's lives. After analysing their assessment reports, it was possible to verify that some types of studies were not considered relevant due to the type of product, previous scientific and clinical knowledge, and the therapeutic intent. However, despite the variation analysed during this study, it can be concluded that there are certain types of non-clinical data that should be presented, namely the proof of concept, which can be in vitro and, if feasible, in relevant in vivo animal models; biodistribution data, to support the pharmacodynamics and the safety of the medicinal product, which can be derived from dedicated biodistribution studies or generated through endpoint integration in other type of studies; and toxicology data, on a case-by-case basis.

In the end, this thesis enabled the analysis of the complexity of CAR T-cell therapies, how the regulatory entities are adapting their assessment and their guidelines to meet the challenges that come with these novel advanced medicinal products, and how the risk-based approach can improve the relevancy and the quality of non-clinical data packages. However, despite all the mentioned remarkable efforts developed for the regulatory pathways of medicinal products, these efforts are still overshadowed by the prices of advanced therapies, hence a more active role of the regulators in the discussion of plausible prices could be crucial.

Conclusively, the approval of COVID-19 vaccines, such as Astrazeneca, put the development and evaluation of medicines in the spotlight, with many people wondering how such a fast process was possible. The fact is that years of research of this type of vaccines had already happened, and the researchers and the regulators had access to these data. For advanced therapies, it should also be expectable that as more and more knowledge is generated, more efficient the development will be, more ATMPs will be approved and, ultimately, more lives will be saved.

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