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

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Preface

An overview will be performed on the currently approved T-cell based ATMPs. The challenges behind the conception of meaningful nonclinical development programs will be identified and the existing regulatory guidance, european and from FDA, will be scrutinised and compared, as well as their applicability to the products currently available in the market.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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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: CAR T-Cells, Cancer Immunotherapy, Marketing Authorisation Application, Biologics License Application, Non-clinical Studies, Advanced Therapy Medicinal Products, Gene Therapy, Risk-Based Approach, European Public Assessment Reports

Resumo

À medida que a medicina evolui, também a complexidade de novas terapias aumenta. Doenças que não tinham cura podem agora ser tratadas com sucesso. Este é o caso de vários cancros hematológicos recidivantes ou refratários que, atualmente, já podem ser tratados com células CAR-T. Esta terapia aproveita o poder imunogénico das células T e veio revolucionar o campo da imunoterapia personalizada. Apesar dos desafios que enfrentam, várias terapias com células CAR-T foram aprovadas pela Agência Europeia do Medicamento e pela *Food and Drug Administration*. Esta tese tem como objetivo explorar como os programas disponibilizados por ambas as entidades reguladoras, bem como a flexibilidade demonstrada durante a avaliação dos medicamentos, especialmente no que diz respeito ao desenvolvimento não-clínico, influenciaram o caminho percorrido por estas terapias avançadas, utilizando o software MAXQDA para facilitar a análise. Inicialmente, foi feita uma comparação entre o percurso de aprovação da EMA e da FDA, seguida de uma análise dos requisitos não-clínicos que as células CAR-T, sendo produtos celulares de terapia genética, devem cumprir. Finalmente, foi incluída uma análise comparativa dos relatórios de avaliação de dois produtos de células CAR-T, dois outros produtos de terapia genética e uma vacina COVID-19, todos baseados na tecnologia de vetores virais para induzir a modificação genética desejada, permitindo demonstrar a importância de uma avaliação de risco durante o desenho de estudos não-clínicos. Os resultados permitiram ainda uma discussão sobre os objetivos comuns dos estudos não-clínicos antes do início dos ensaios clínicos e o uso de modelos animais relevantes.

PALAVRAS-CHAVE: Células CAR-T, Imunoterapia, Autorização de Introdução no Mercado, Estudos não-clínicos, Medicamentos de Terapia Avançada, Terapia Genética, Relatório Público Europeu de Avaliação

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List of Abbreviations

ADME	Absorption, Distribution, Migration and Excretion
APC	Antigen Presenting Cells
ATMP	Advanced Therapy Medicinal Product
BLA	Biologics License Application
CAT	Committee for Advanced Therapies
CHMP	Committee for Medicinal Products for Human Use
CTL	Cytotoxic Lymphocytes
COVID	Coronavirus Disease
DLBCL	Diffuse Large B-cell Lymphoma
EMA	European Medicines Agency
EPAR	European Public Assessment Report
EU	European Union
FDA	Food and Drugs Administration
GTMP	Gene Therapy Medicinal Product
GVHD	Graft versus Host Disease
MAA	Market Authorisation Application
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
NHL	Non-Hodgkin Lymphoma
PD	Pharmacodynamics

r/r	Refractory or Relapsed
scFv	Single Chain Variable Fragment
SLO	Secondary Lymphoid Organ
TAA	Tumour Associated Antigen

1. Introduction

The landscape of the pharmaceutical industry is constantly growing and changing. There are new drugs and therapies reaching the market almost every day and the coronavirus disease (COVID)-19 pandemic showed how fast this industry can be when there is a medical emergency worldwide.

Usually, most medicines take approximately 12 years[1] to go through the development process until they reach the market, with a lot of initially promising ones being left behind. However, this is a high-risk high-reward venture considering that the pharmaceutical industry is a trillion-dollar business[2]. Recently, the vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been in the spotlight, raising many questions regarding the regulatory process of medicines and how their safety and efficacy is tested before being administered in humans.

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. The field of personalised medicine has been evolving and changing the treatment options for patients with many different conditions and diseases. Furthermore, it allows each medicinal product to be tailored to each patient, enhancing the effectiveness and the outcome. However, in a world moved by profit, it can be challenging for pharmaceutical companies to produce a specific product for each patient at a price accessible enough for healthcare systems, insurance companies and patients themselves. Additionally, the non-clinical and clinical tests of this type of medicines cannot be the same has “one size fits all” drugs, so regulatory agencies 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. Cancer is characterized by uncontrolled cell growth, resistance to apoptosis, insensitivity to growth suppressors, sustained angiogenesis invasion and metastasis. It is also associated with genome instability, inflammation, and immune evasion [3]. In 2020, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred worldwide, and these numbers are only expected to grow in the future[4]. Cancer treatment is commonly associated with chemotherapy, radiotherapy, and tumor removal surgery, but these are only a portion of the existing therapies nowadays. In fact, cancer therapies can be grouped into non-immunotherapies, where the aforementioned therapies are included, and immunotherapies, treatments that directly modulate the patient’s own immune system to achieve beneficial clinical outcomes [5]. Even though the likelihood of patients reaching a cancer-free state has been improving, cancer is and will continue to be one of the leading causes of death in the developed world. Furthermore, there is still a lack of effective cancer treatments (especially after remission) in some types of cancer, and the variability of outcomes exhibited when individuals with the same disease respond differently to the same therapy is a concern for doctors and patients. All these factors justify the intense research of cancer treatments tailored to each patient. In recent years, cancer immunotherapy has been emerging and exciting not only cancer researchers, clinicians, and patients, but also

pharmaceutical companies. Immunotherapy was actually the subject of the 2018 Nobel Prize in Physiology or Medicine for its favourable efficacy in cancer treatment [6], which helps to understand the importance it can have in medicine. Targeted immunotherapies give the patient's immune system a fighting chance against cancer cells and show a promising future for the field of personalised immunotherapy.

But if cancer cells are so elusive, how can a patient's debilitated immune system fight them? Researchers have long understood the essential role of T lymphocytes in the immune system response to cancer cells. Harnessing their power to treat cancer patients in the form of adoptive cell therapy (ACT) was first developed in the early 1990s, with the first chimeric antigen receptor (CAR) being conceived by Eshhar et al[7]. The use of gene transfer technology enabled the engineering of T cells to express CARs, which are capable of specifically recognising their target antigen in cancer cells. The first approval of a CAR T-cell therapy by the FDA and the EMA, in 2017 and 2018, respectively, represented a big step in 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. Despite showing remarkable results, CAR T-cell therapy still faces many challenges, both in the manufacturing and regulatory process as well as in its administration to patients in the hospital setting[8].

CAR T-cells belong to the category of advanced therapy medicinal products (ATMPs) in Europe and Cellular & Gene Therapy Products in the United States (US), which are required to follow multiple regulatory guidelines, in particularly, regarding the non-clinical 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.

With all this in mind, the present thesis was divided in three major topics, which include the characterisation of CAR T-cells, drug development and non-clinical studies design.

The Chapter 2, *Background*, intends to explain the rationale behind T-cell based therapies and the type of challenges they face, the development process followed by medicinal products from the lab to the market, including the different type of programs provided by the EMA and the FDA, and a summary of the risk-based approach.

In Chapter 3, *Methodology*, the methods used for the studies presented in this thesis are described, including the use of the MAXQDA software, that assisted the analysis of multiple regulatory documents from the EMA and the FDA, as well as the comparative analysis of the assessment reports belonging to CAR T-cell products and other relevant medicinal products.

During Chapter 4, *Results*, various studies are presented in a step-by-step approach, allowing to use information obtained from the first steps as basis for the following steps. This chapter enables the comparison of supportive programs and timelines for accelerating development and approval of CAR T-cells in the EU and US and allows to understand the non-clinical development required for this therapy. Furthermore, a case study like exercise was conducted to compare the non-clinical studies that supported the approval of two CAR T-cell products, two other gene therapy products and a COVID-19 vaccine.

2. Background

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

2.1.1. T-cells in Immune Response

The immune system is a vital part of human survival and it is the primary defense mechanism of the body against cancer cells. The immune system comprises the innate immune system and the adaptive immune system. The innate immune system has a quick and broad response in the event of viruses, bacteria, fungi, and even non-infectious problems, typically leading to inflammation, and to the activation of the adaptive immunity. The innate immune cells recognise general danger- or pathogen-associated patterns through their Toll-like receptors (TLRs). On the other hand, the adaptive immune system cells are T and B lymphocytes, which 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 of producing neutralising antibodies. T lymphocytes originate from haematopoietic stem cells in the bone marrow and then mature in the thymus gland (whereas B lymphocytes mature in the bone marrow) and can be divided into two groups – CD8+ T-cells and CD4+ T-cells. CD8+ T-cells are usually cytotoxic lymphocytes (CTLs), which are crucial for recognising and eliminating virus-infected cells and cancer cells through apoptosis. A CD4+ T-cell can be a T helper cell (TH), that produces and secretes different molecules to coordinate and activate other cells, or a regulatory T-cell (Treg), that monitors and inhibits the activity of other T-cells. Besides these types of adaptive cells, there is also memory B and T-cells that upon re-encountering their specific pathogen, can immediately induce a neutralising immune response.[9][10]

During the 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 [9].

Adaptive immunity depends on specific recognition by a TCR of an antigenic peptide in the presence of an MHC molecule. This allows TCRs to discriminate foreign antigens (from pathogens or tumours) from self-antigens. The TCR is a transmembrane heterodimer consisting of two chains (usually, α and β) linked by a disulphide bond. Within these chains are complementary determining regions which determine the (unique) antigen to which the TCR will bind, only in the context of an appropriate class I

or class II MHC binding. Each TCR associates with either a CD8 or CD4 coreceptor that binds to a nonpolymorphic region of MHC class I or II, respectively, which further stabilises the interaction between the T-cell and the APC. The TCR is noncovalently associated with a complex of proteins known as CD3 (composed of γ-, δ-, ε-, and ζ-subunits), and it is the cytosolic region of this complex that is responsible for propagating the intracellular signal following TCR ligation (figure 1), leading to proliferation and differentiation of the naïve T cell into an effector cell[11],[12],[13].

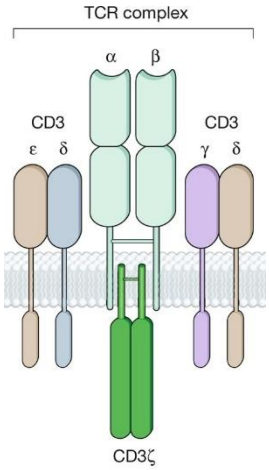


Figure 1 - Schematic of the T cell receptor and associated molecules important for TCR recognition and signal transduction. Credit: [14]

When the immune cells encounter a tumour, once the T lymphocytes differentiate and migrate to the tumour site, the activated CD8+ T-cells produce large amounts of proinflammatory cytokines and exhibit a profound tumour-directed cytotoxicity. Stimulated CD4+ T-cells secrete various cytokines that promote the differentiation of B cells into antibody-producing plasma cells, enhance the capacity of dendritic cells to induce CD8+ T cell responses, and can eliminate tumour cells directly [15].

It is important to notice that, in a normal immune response, the T-cells die off after the problem is resolved. However, exaggerated T-cell immune responses from any of the T-cell subsets results in a range of immunopathologies from autoimmunity to allergy and cancer. This latter usually occurs when there is an unbalanced response of regulatory T-cells [9], which excessively limits the CTL response to tumour cells.

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.

2.1.2. Engineering T Lymphocytes for cancer

Harnessing the power of T lymphocytes specificity has led to a new generation of extremely promising immunotherapies. Different types of T-cells can and are being explored for different therapies. For instance, CTLs can be used to target tumour cells after being engineered to express receptors such as TCRs or CARs, in addition, CTLs specific for viruses can be purified *ex vivo* and reinfused into patients transplanted with haematopoietic stem cells to help combat viral reactivation. On the other hand, Tregs

can also be expanded *ex vivo* and used in the treatment of autoimmune diseases, allergies, transplant rejection and graft-versus-host disease (GVHD), and, more recently, even in non-immune diseases, such as neurological disorders and tissue repair[16].

These types of immunotherapies are defined as adoptive cell therapy (ACT), which 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[8]. The present work focuses on CAR T-cells, as these are the main component of recently approved medicinal products.

In fact, both TCR and CAR T therapies modify the patient's own T lymphocytes *ex vivo* and then infuse them back into the patient's body to kill cancer, but their mechanisms for recognising antigens are quite different. Recombinant TCRs are generated through the integration of the genes that encode for the α- and β-chains specific for an antigen of interest, which is then recognised in the context of the MHC. CAR T-cells use antibody fragments that bind to specific antigens on the surface of cancer cells and have the ability to recognise whole proteins expressed in target tissues, rather than being restricted to antigens presented in the context of the MHC. Notably, TCR-T cell therapy can have a wider range of targets as they can detect intracellular antigens, and may be more adequate for solid tumours, for example, whereas CAR T-cell therapy has the advantage of being MHC independent [17],[18].

CARs are genetically engineered receptors that mimic TCR activation, redirecting specificity and effector function towards 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 [19].

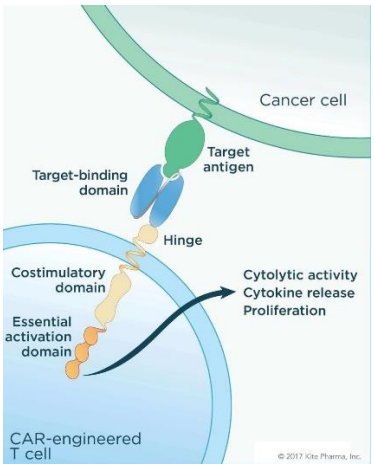


Figure 2 - The structure of CARs*:* The engineered receptor on CAR T-cells binds to an antigen on cancer cells. After binding, components of the receptor inside the T cell provide signals that activate it. Credit: Kite Pharma[20]

Antigen recognition and binding domain

The antigen-binding domain is typically a single-chain variable fragment (scFv) molecule derived from a monoclonal antibody (mAb) that recognises and binds to the tumour-associated antigen (TAA) present on the cancer cell surface[21]. The scFv is typically composed of variable heavy and variable light chains

of the mAb, connected by a flexible linker. scFv affinity for the target antigen must enable the recognition of tumour cells and T-cell activation, however, excessively high affinity might result in activation-induced cell death of the CAR-expressing cell, exhaustion, and possible toxicities, lowering the efficacy and safety of CAR T-cell therapy[19].

Hinge and transmembrane domains

The hinge provides flexibility and facilitates access to the target antigen. Its length and composition can affect antigen binding and signalling through the CAR. Amino acid sequences from, for example, the cluster of differentiation (CD)8 and CD28 have been used in CAR hinge domains.

On the other hand, the CAR's transmembrane domain, which is commonly derived from type I proteins such as CD3, CD28, CD4, or CD8, “anchors” the CAR in the T-cell membrane and influences the stability and function of the CAR[19].

Intracellular signalling domain

The intracellular signalling domain generally contains a T-cell activation domain derived from the CD3ζ chain of the TCR, as well as, in the case of second and third generation CARs, costimulatory molecules such as CD28 and CD137 (also called 4-1BB), which enables the cells to obtain long-lasting *in vitro* proliferation capacity and strong cytokine secretion ability [8]. CARs without the costimulatory domains (i.e., first generation CARs) are insufficient to induce productive T cell responses and lead to limited *in vivo* T-cell persistence and activity [22]. T-cells with CARs containing costimulatory domains in addition to activation domains produce interleukin (IL)-2 and can proliferate upon repeated antigen exposure. Different costimulatory domains can lead to functionally different immune responses. Studies suggest that CD28-based CARs have greater initial antitumor activity, whereas 4-1BB signalling enhances CAR-T cell persistence and reduces exhaustion[23], [24]. The addition of either of these costimulatory domains in second generation CARs was a major improvement for CAR T-cell products that have been approved by the regulatory entities[22].

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 this therapy, broaden the range of cancers responsive to such treatments and facilitate more rapid, reliable, and efficient production of these products.

2.1.3. 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[17]. Notably, this therapy does not

replace a patient's entire hematopoietic system as, for example, bone marrow transplant, and instead attempts to augment the immune system by the addition of these engineered T-cells. The steps of this therapy are summarised in figure 3.

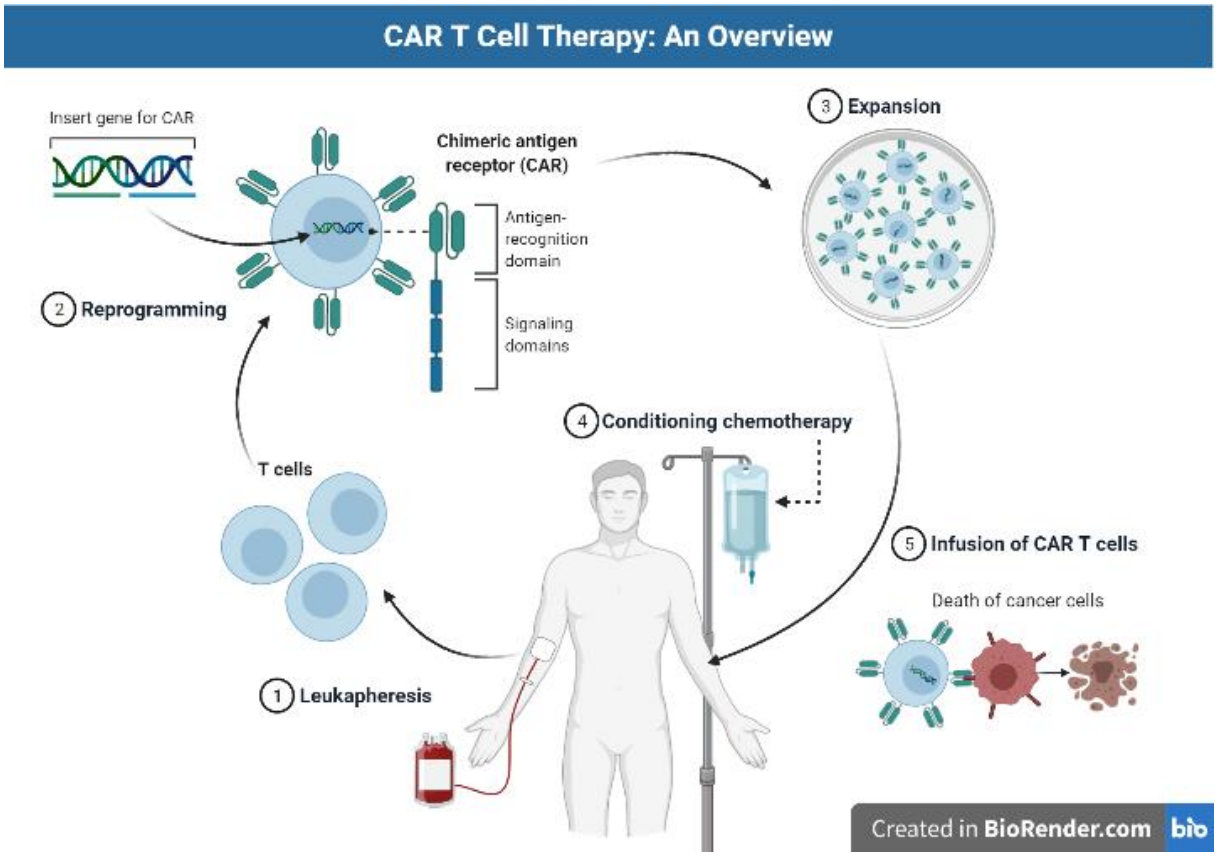


Figure 3 - Schematic representation of the process followed for CAR T-cell administration

Transforming the T-cells from the patient into CAR T-cells that target specific markers requires the delivery of genetic instructions. This gene editing is most commonly performed with transduction techniques (e.g., via viral vectors), but, more recently, genome editing tools such as CRISPR-Cas9 have also begun to be used[18], [25].

Adoptive transfer of autologous CD19-targeted CAR T-cells became the first therapeutic approach with a genetic engineering component to be approved by the FDA for use in the US[26]. 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[27]. The types of haematologic cancers that have showed notable antitumor effects after treatment with (CD19-specific) CAR T-cell therapy are mentioned below. It is worth noticing that this therapy is not used as a first line of treatment for new cancer patients, but rather in case of refractory or relapsed (r/r) forms of the disease.

Acute lymphocytic leukaemia

The initial development of CAR T cell therapies has focused largely on acute lymphocytic leukaemia (ALL), the most common cancer in children. Leukaemia affects the blood and bone marrow. In ALL, lymphocyte stem cells become immature white blood cells called lymphoblasts. These leukemic

lymphoblasts do not function normally and block the production of normal cells while also growing and surviving better than normal cells. This causes a depletion of healthy blood cells (erythrocytes, leukocytes and platelets) and may lead to life threatening conditions [28]. Even though more than 80% of children diagnosed with ALL that arises in B cells—the predominant type of paediatric ALL—will be cured by intensive chemotherapy, for patients whose cancers return after chemotherapy or a stem cell transplant, the treatment options were almost non-existent, making relapsed ALL the leading cause of death from childhood cancer. Even though ALL incidence in adults is quite lower, both younger and older adults show really poor survival rates (5-year relative survival is under 50% even among adults 20–24 years, and decreasing with age), which shows the public health burden of this disease and a substantial unmet medical need[29].

Non-Hodgkin lymphoma

In lymphoma, cancer develops in the lymphatic system, when lymphocytes start growing abnormally. Eventually, these cells outnumber the healthy cells and prevent the immune system from working correctly. As they accumulate, they can form tumours throughout the body. About 85-90% of Non-Hodgkin lymphoma (NHL) cases start in the B cells[30]. NHL has many different subtypes, among them are:

- Diffuse Large B-Cell Lymphoma (DLBCL) – the most common form of lymphoma, represents around 30–40% of all cases of NHL. R/R DLBCL is a major cause of morbidity and mortality with a median survival of around 6 months[31].
- Primary mediastinal large B-cell lymphoma – rare form of NHL, represents approximately 2–4 % of all NHL, predominantly occurs in adolescents and young adults. It is a subtype of large B-cell lymphoma that is derived from a thymic B cell[20].
- Mantle cell lymphoma (MCL) – rare subtype, usually occurs in middle-aged or older adults. It develops in the part of the lymph node called the mantle zone, hence the name[32].

2.1.4. 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, including antigen loss 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.

Antigen Loss and Suboptimal persistence in vivo

CAR T-cells have the potential to replicate *in vivo*, and long-term persistence can lead to sustained tumour control, however, there is still the risk of relapse. Data relative to ALL shows a complete remission rate of ALL in CD19 or CD22 CAR T-cell treatment reaching 57% to 93%, and, at the same time, a relapse rate reaching 14% to 66% [33]. There are two main reasons that can constrain the durability and hence efficacy of CAR T cell therapies: antigen loss and suboptimal persistence of CAR T-cells *in vivo*. Antigen loss is the complete or partial loss of target antigen expression by the cancer cells, and it

has been observed in a significant proportion of patients treated with CAR T-cells. For example, reports from multiple CD19 CAR trials for ALL have indicated that 7 to 25% of patients treated with this CAR T cell therapy relapsed with $CD19^-$ disease. CD19 loss can occur via two distinct mechanisms: 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, most often acute myeloid leukaemia[34]. To address this issue, researchers have developed CAR T-cells to target multiple antigens—for example, both CD19 and CD22—to prevent this "escape" by cancer cells. The suboptimal persistence of CAR T-cells *in vivo* is the second main reason that CAR T-cells sometimes fail to provide long-term protection and it occurs when the cells become "exhausted," which can allow any residual cancer cells to proliferate and lead to a relapse. To address this, several strategies to engineer "exhaustion-proof" CAR T-cells are being explored. The strategy of using T-cell populations with higher percentages of less differentiated T cell subsets that have a greater proliferative capacity[14], [19] or the combination of CAR T-cells with other immunotherapies, particularly the checkpoint inhibitors that block the PD-1/PD-L1 pathway, which can lead to T cell exhaustion[18] are promising approaches.

Solid tumours

Another known limitation is the lack of efficacy that CAR T-cell therapy has in the treatment of solid tumours. In fact, most clinical trials on CAR T-cells in solid tumours indicate poor responses and severe toxicities caused by on-target off-tumour antigen recognition[35]. While CD19 serves as an effective target antigen because it is expressed almost universally in the B cell lineage, when it comes to solid tumours, there is a lack of suitable tumour-specific binding, due to tumour's antigen heterogeneity. Thus, a great deal of ongoing work is seeking to identify more suitable targets for CAR T-cells in a range of solid cancers. In addition, solid tumours often possess a hostile tumour microenvironment that can block, deactivate, or repel immune cells[18]. Currently, the inefficient T cell trafficking, suboptimal antigen recognition specificity, immunosuppressive tumour microenvironment – physical barriers and T cell inhibitory signals – are considered the main obstacles in solid tumour CAR T therapy[36].

Toxicities and other side effects

Although CAR T-cells can be extremely effective in killing malignant cells, they can also cause dangerous side effects, including systemic cytokine toxicities and on site/off-tumour toxicities[37].

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 other 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[19].

CRS is a systemic inflammatory response that has been observed in other types of T-cell directed therapies, being the most commonly observed toxicity associated with CAR T-cell therapy. CRS is triggered by the activation of T-cells following the engagement of the CAR with the TAA and it is "*characterized by increased serum levels of inflammatory cytokines, fever, hypotension, hypoxia, and*

organ dysfunction"[19]. It is becoming increasingly significant with the use of immunotherapies that increase immune activation to levels greater than that occurring in nature. Its severity ranges from mild to severe and can eventually lead to patient death. The occurrence and severity of CRS varies between CAR T products and depends on the malignant disease. For example, CAR T-cells with CD28 costimulatory domains tend to cause a higher incidence of all-grade and severe CRS than CAR T-cells with 4-1BB costimulatory domains, moreover, CRS is generally less common and less severe in non-Hodgkin lymphoma than in ALL[37]. Depending on the grade of CRS, it may require only symptomatic management, and, in higher grades, it is often responsive to treatment with the anti-IL-6 receptor antibody tocilizumab, the anti-IL-6 antibody siltuximab or corticosteroids[38].

Clinical trials of CD19 CAR T therapy have all reported a significant incidence of neurotoxicity regardless of the CAR constructs, patient population, or disease subtype[39]. After the report of the new American Society for Transplantation and Cellular Therapy consensus recommendations in 2019, the term ICANS was adopted[40]. ICANS is associated with disruption of the blood–brain barrier and increased cerebrospinal fluid cytokine levels. It can be defined as "a disorder characterized by a pathologic process involving the central nervous system following any immune therapy that results in the activation or engagement of endogenous or infused T-cells and/or other immune effector cells". The symptoms and signs of this syndrome can be progressive and may include somnolence, disorientation, aphasia, altered level of consciousness, impairment of cognitive skills, motor weakness, seizures, and cerebral oedema, often occurring concurrently with or following CRS[40], [41]. Management of neurotoxicity may involve only supportive measures, IL-6 pathway inhibitors if symptoms of CRS are also present, or, for higher grades, the use of corticosteroids, as well as appropriate treatment of neurological symptoms and abnormalities evident on neuroimaging and electroencephalography[41].

On-target/off-tumour effects are another serious safety concern in CAR T therapy. Most TAAs are expressed not only on tumour cells but also on healthy cells, although often at much lower levels, which means that CARs can bind to healthy cells leading to their death. This widely observed toxicity of anti-CD19 CAR T-cells leads to B-cell aplasia and is well tolerated and treatable with periodic infusions of intravenous immunoglobulins to replace antibodies that would otherwise have been produced by the patient's healthy B cells. To overcome the on-target off-tumour effect, researchers have been studying the possibility of tuning receptor expression or affinity to discriminate between the high and low antigen expression profiles found in malignant and normal cells, respectively, using lower affinity scFv CARs, for example. There are also combinatorial antigen recognition strategies being explored, which increase the specificity of CAR T-cells for tumour cells and reduce the off-tumour effects as well as the antigen escape. These options illustrate the profound effects that subtle changes in the scFv can have on the therapeutic window of CAR T-cells[19], [41].

There are plenty of strategies to construct CARs that can prevent these toxicities. One approach can be the incorporation of suicide genes into the CAR construct (e.g., inducible caspase 9 (iCasp9)) that, if necessary, upon administration of a small molecule, lead to the apoptosis of CAR T-cells[42]. The incorporation of an elimination marker into the CAR construct may also be used to manage toxicity. Another strategy is the incorporation of an on-off switch, which enables the reversible pharmacological

control of these cells[41]. None of the approved CAR T cell therapies use this type of strategies to manage the common toxicities mentioned above, however, fourth generation CARs are already designed with additional molecular elements that increase safety and controllability[43].

Manufacturing Challenges

Transforming the patient's blood components into the therapy that is eventually infused into the patient requires a highly complex manufacturing process, which is long, expensive, complicated, and highly regulated[44]. The current approved CAR T cell products as well as the majority of those used in clinical trials to date are patient-specific since they are manufactured using autologous T-cells. Although this specificity can make CAR T-cell therapy very efficient (and persistent), it comes with a few hurdles. First, harvesting sufficient numbers of functional T-cells from patients with cancer can be difficult, as a consequence of the disease or previous cancer treatments. Second, autologous CAR T cell products have a lengthy manufacturing process (around 4 weeks) that might not be feasible for patients with advanced-stage cancer, in whom the disease might progress during manufacturing. Furthermore, the logistics of the process are quite challenging, from conducting a successful leukapheresis, accessing a manufacturing and a treatment facility, and lastly to shipping the CAR T-cells, all of which have associated costs[45].

These therapies are very intensive in terms of skilled labour, reagents, and facilities[19] and few laboratories and centres have the ability to make CAR T-cells, which makes conducting human clinical trials more challenging and also limits their use on a larger scale. Recently, the National Cancer Institute (NCI) team decided to use a "closed" system for manufacturing CAR T-cells, called Prodigy. This system is "closed" meaning that once the collected cells from the patient are put into the system, it does not require more labour, producing a completed CAR T-cell product within 2 weeks. With a closed system, the only variable to account for is the patient cells going in and it might facilitate the access to more laboratories and centres[44].

Indeed, one of the main challenges of this largely personalised medicine is the development of efficient technologies and cost-effective clinical manufacturing platforms to support the later clinical trial phases and ultimately commercialization[46].

Burden on Health Care Systems

ATMPs are still novel therapies, and the cost of CAR T cell products, which can go from $373,000 to $475,000 US dollars per patient, is exceedingly high, which further limits its availability and represents a major challenge for health care systems worldwide[47].

It is vital to ensure that the incremental benefit of the novel ATMP is proportionate to its incremental cost above conventional therapies in order to maximise the possibility of getting reimbursement at a commercially feasible price point. In this regard, populations with a high disease burden and unmet medical needs may be the preferred choices, since the potential for improving patient benefit and profiting from healthcare cost offsets is larger.[47]. Outcome-based risk-sharing schemes together with real-world evidence provide opportunities to manage uncertainty and reward for the full benefits of the

ATMPs. Such schemes in combination with annuity-based payments can also help minimise payers' concerns. For example, in Spain and Italy, the payment of two CAR T cell products, Yescarta and Kymriah, is done in instalments linked to individual patient outcomes, so the companies that manufacture the products only get paid if the agreed outcome(s) has been achieved and sustained[48] (figure 4).

	Italy	Spain
Reimbursement scheme	Outcomes-based staged payments	Outcomes-based staged payments
Detail	Payments in three instalments linked to individual patient outcomes	Payments in two instalments linked to individual patient outcomes
Key outcomes considered	Specifics not disclosed	Complete response Survival
List price	Kymriah: €300,000 [36] Yescarta: €327,000 [37]	Kymriah: €320,000 [80] Yescarta: €327,000 [81]

Figure 4 - Summary overview of list prices, reimbursement schemes and key outcomes associated with the reimbursement of CAR T cell therapies in Spain and Italy[48] (adapted)

In Portugal, according to the *2019 Report of INFARMED*[49], in the Pharmacotherapeutic Group of Antineoplastic and Immunomodulators Agents / *Medicamentos Antineoplásicos e Imunomoduladores*, there were 32 (not including generic medicines) of these medicinal products in the level of reimbursement A (which equivalates to a 90% reimbursement) and 82 in the level of reimbursement C (which equivalates to 37%). The expenditure distribution of Sistema Nacional de Saúde (SNS) regarding the Group of Antineoplastic and Immunomodulators Agents is 0,58%. As a comparison, the expenditure the Pharmacotherapeutic Groups of Endocrine System, Central Nervous System and Cardiovascular System was 26,48%, 20,20% and 17,11%, respectively. Which means that the funding of cancer treatments is still low when compared to other types of diseases, even though the cost of these treatments, especially with the rise of personalised immunotherapies, tends to be very high.

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.

2.1.5. Allogeneic CAR T cell therapy

A shift from autologous to allogeneic donor T-cells (where the cells are taken from a non-related healthy donor rather than from the patients themselves) could surpass some of the biggest limitations discussed above but it also comes with new challenges of its own, figure 5.

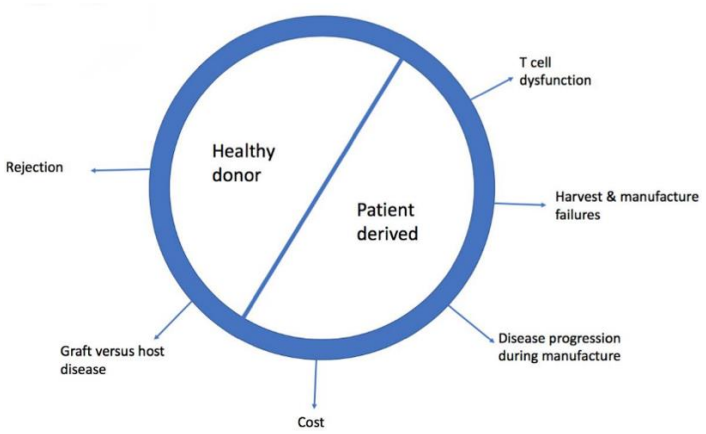


Figure 5 - Challenges of using healthy donor versus patient derived CAR T-cells[50] (adapted)

As it was mentioned before, the manufacture of autologous CAR T-cells is time consuming, logistically challenging, and expensive. Collecting T-cells from patients with reduced levels of T-cells and/or suboptimal T cell states and from patients with rapidly proliferative disease justify why it might not always be possible to manufacture an autologous CAR T-cell product[51].

Allogeneic CAR T cell therapy mainly uses T-cells derived from peripheral blood mononuclear cells and, rarely, from umbilical cord blood[52]. Another promising option is to use T-cells derived from induced pluripotent stem cells (iPSC)[53], which usually have higher proliferation capacity. The use of different T-cell sources and the manufacturing process of allogeneic CAR T-cells is demonstrated in figure 6.

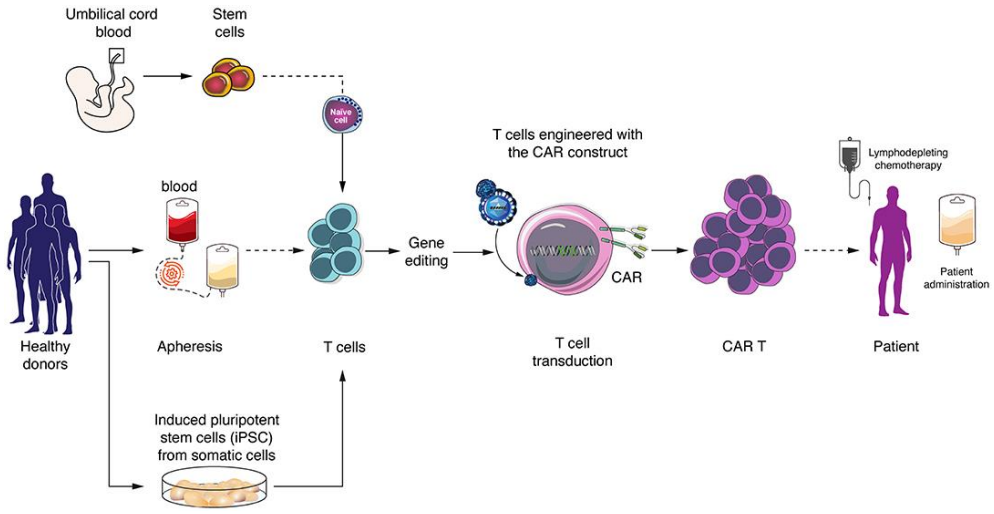


Figure 6 - Generation of allogeneic CAR T-cells from different sources of T-cells: peripheral blood mononuclear cells from healthy donors, umbilical cord blood, and derived from induced pluripotent stem cells (iPSCs). After the gene editing step, the cells follow a similar manufacturing process as autologous CAR T-cells. Credit: [54]

There are two major issues when using donor cells – graft versus host disease (GVHD) and human leukocyte antigen (HLA) incompatibilities[51]. GVHD occurs when the donor's T-cells perceive the patient's healthy cells as foreign, attacking and destroying them, which can, in severe cases, be life-threatening. On the other hand, the host's immune cells can also recognise and eliminate the donor T-cells, restricting their antitumour activity. HLA is the genetic designation for the human MHC, and it may be incompatible between the donor and the recipient. HLA disparities are associated with higher risk of

graft failure, delayed immune reconstitution and GVHD, therefore, the donor's HLA type should match the HLA of the patient.[54].

To overcome the obstacles of GVHD and HLA incompatibilities, gene editing technologies have already resulted in strategies to disrupt the endogenous TCR–αβ of the CAR T-cells (αβ TCR is the determinant of T cell alloreactivity), lymphodeplete the host immune system to minimise rejection, and engineer the CAR T-cells to be resistant to lymphodepleting drugs[51].

Ultimately, the development of universal off-the-shelf CAR 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[27], [50], and potentially reducing the burden on healthcare systems.

2.2. The approval journey of medicinal products

2.2.1. Overview of Drug Development

The complete process of drug development goes from early drug discovery with basic biological research, disease modeling, 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[55]. These steps are summarized in figure 7.

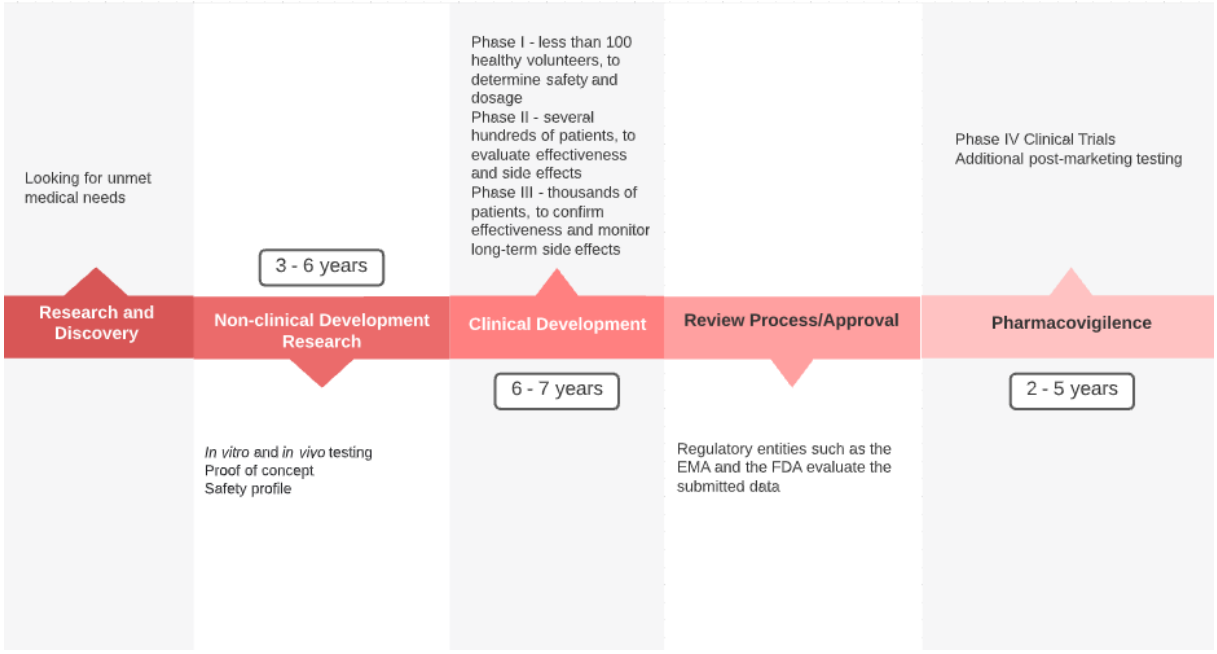


Figure 7 - Overview of the drug development process

Research and Discovery

In the early stages of developing a new medicine, researchers usually start with basic research to understand the processes behind a specific disease and manufacturers focus on unmet medical needs or the possibility of overcoming the performance of already existing medicines. In this stage, a lot of trial and error needs to happen until a promising group of therapies emerges and progresses to the following steps[1]. Pharmaceutical and biotechnology companies, both big and small, as well as doctors and academics, investigate tens of thousands of new substances each year, but only a few hundred reach the non-clinical development stage. All pharmaceutical companies invest a percentage of their profits in R&D as this allows them to continuously renovate the medicinal products they offer. It is also important for the government to allocate a percentage of its budget to scientific investigation as this gives the necessary resources for researchers to continue innovating in the medical field.

Non-clinical Development

Before any new treatment is given to humans, it goes through a process of pre-validation, where safety and efficacy tests are conducted using *in vitro*, *ex vivo* and *in vivo* models. *In vitro* tests are usually performed for the initial evaluation of biological activity, they can be based on a monolayer (2D) cell culture or 3D cell culture. *Ex vivo* tests often involve living cells or tissues taken from an organism into an artificial environment outside the organism with the minimum alteration of natural conditions. *In vivo* studies are usually performed after *in vitro* and *ex vivo* studies and require the use of animal models. The animal models used depend on the type of disease/condition as well as on the type of medicinal product being studied, and their use must always be well documented and justified[21]. Furthermore, some studies must be performed under Good Laboratory Practice, ensuring the quality and reliability of the pre-clinical studies, as well as allowing a uniformised review process and traceability of data[1]. Notably, after the safety and efficacy of the medicinal product is demonstrated, some non-clinical studies can be conducted in parallel with clinical trials.

The three main groups of non-clinical studies are: 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[1]. There are several specific sub-studies within these three categories that will be explored in a following chapter.

Clinical Development

After sufficient non-clinical data is collected and the medicinal product has proven to be safe and effective enough to be tested in humans, the applicant may request the authorisation to start clinical trials. The clinical development should enable a benefit/risk assessment, based on the specific characteristics of the product, the target population, and existing treatments[56].

There are three different types of clinical trials, which differ on the goal of the study and on the number and type of participants. Usually, the phases of clinical development are conducted one after the other,

and the information acquired from previous studies is used to determine the next steps in the medicine evaluation.

Phase I clinical trials usually include less than a hundred healthy volunteers. However, the selection of participants for these studies depends on the type of medicinal product. For cancer therapy, for example, researchers usually conduct phase I studies in patients with the type of cancer in question.[57] These studies allow to evaluate safety and estimate the optimal dosage to be administered. The volunteers are closely monitored in order to gather important information about the interaction of the medicinal product with the human body. The dosage is adjusted by evaluating the tolerability and the occurrence of side effects. Usually, there are dose escalation studies, which means that the first group of patients receives a smaller dose, which is then increased in the following groups[56]. The goal is to determine the best dose and administration scheme to limit risks and maximize possible benefits, helping the design of phase II studies[57].

In Phase II clinical trials, a few hundreds of patients that suffer from the condition/disease in question enrol to determine the effectiveness of the new medicine, for example, through the measurement of the objective response rate. The effects of the new medicine are often compared with different treatments, either a placebo or the standard treatment available at the time. Due to the small number of patients that are enrolled in these clinical trials, their purpose is more to provide researchers with additional safety data [57].

Phase III studies are not always required for marketing approval. They typically involve thousands of patients that suffer from the condition/disease, gathering even more information and confirming safety and effectiveness across different populations. They have longer duration, which allows to assess long-term or less common side effects. The goal of phase III studies is to demonstrate whether the medicinal product offers a real treatment benefit to a specific population[56].

2.2.2. Pharmacovigilance

After the therapy is approved by the regulatory entities, it reaches the post-approval life cycle management and pharmacovigilance. In this stage, phase IV clinical trials may be conducted in order to assess the long-term effects of the therapy. Additionally, pharmacovigilance programs guarantee that the purpose of the medicine is being fulfilled and monitor safety issues under real world conditions. Furthermore, usually, the applicant is required to submit periodic safety updates to the regulators[55].

2.3. Regulatory Framework in the US and the EU

There are two main regulatory entities worth mentioning to better understand the journey of a new medicinal product – the EMA and the FDA. One is responsible for authorising the marketing of a new product in the European Union (EU) and the other in the United States (US), respectively.

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, whereas generics and non-prescription medicines

are assessed and authorised at a national level, by the competent authorities of each country. It is also the role of the national regulatory authorities in which the clinical study is to be conducted to approve the clinical trial application (CTA) after it is submitted by the applicant. In the US, the clinical trial authorisation is given by the FDA, that also performs inspections of the clinical trial study sites and anyone involved in the research[58].

When the quality, non-clinical and clinical data that support the efficacy and safety of the new advanced medicinal product have been collected, 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. However, before the submission of these applications, the regulatory agencies play an important role in helping the applicants reach this stage[58].

Very early in the development stage, the applicant can submit an orphan drug/medicinal product designation request to both regulatory agencies. According to the EMA, orphan medicinal products are for "*diagnosing, preventing or treating life-threatening or very serious conditions that are rare and affect not more than 5 in 10,000 persons in the EU*". For the FDA, this number goes to 200,000 persons in the US[59]. Both the EU and the FDA offer a range of incentives to the developers of these products, such as protocol assistance (for EU only), fee reductions, and a 7- or 10-year market exclusivity, in the case of the FDA and the EMA, respectively[60]. The orphan designation request is a separate process from seeking approval or licensing, its goal is to help ensure that medicines for rare diseases are developed at all[61].

As it was mentioned before, the EMA and the FDA offer scientific advice at crucial timepoints for the medicine's development. In the US, for a new medicine to be used in clinical trials, it must receive the Investigational New Drug (IND) designation, which is granted by the FDA. Hence, the FDA offers advice in a pre-IND meeting and also in the pre-BLA meeting[62]. In the EU, the responsibility of overseeing clinical trials is of the National Competent Authorities (NCA) of each member state, and so the first scientific advice meeting is with the NCAs and the EMA joins in the following up meetings.

In both the US and the EU, the applicant may apply for expedited development programs. The FDA established the Fast Track Designation in 1997, the Breakthrough Therapy Designation in 2012 and the Regenerative Medicine Advanced Therapy Designation in 2017[63]–[65]. The Fast Track Designation is intended for medicinal products that treat serious conditions and have demonstrated the potential to address an unmet medical need, whether in the case of a condition with no current therapy or in the case that the fast track drug brings considerable advantages over the existent[63] giving access to expedite development and review, and making the product eligible for Priority Review (i.e., the review of the BLA is shortened from 10 to 6 months)[66] and Rolling Review (i.e. data is evaluated as it becomes available instead of waiting for all the necessary data to be collected). The Breakthrough Therapy Designation is given to medicinal products indicated for the treatment of serious conditions and that may demonstrate substantial improvement over available therapy on clinically significant endpoints, generally related to irreversible morbidity or mortality and symptoms of the disease, giving access to all the features of the Fast Track Program in addition to intensive guidance on efficient drug development (usually regarding the design of efficient clinical trials)[64]. This is equivalent to the EMA's PRIority

MEdicines (PRIME) Scheme, launched in 2016, which was designed for medicines that offer a major therapeutic advantage over existing treatments or target unmet medical needs[67], 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 210 to 150 days. Finally, the FDA's Regenerative Medicine Advanced Therapy Designation is intended for regenerative medicine therapies that target serious or life-threatening diseases and for which the preliminary clinical evidence shows potential to address unmet medical needs, giving access to all the Breakthrough Therapy features in addition to addressing potential ways to support accelerated approval and satisfy post-approval requirements[65].

When it comes to granting marketing authorisation, the FDA has two different possibilities and the EMA has three. When there is comprehensive clinical data at the time of the BLA/MAA, a positive benefit-risk balance, and a significant demonstration of safety and efficacy based on a therapeutically relevant endpoint(s) or when extensive clinical experience has been gained, both the FDA and the EMA grant a standard marketing authorization[58]. In the US, this is the final step for the medicine's approval, but in the EU, the ultimate decision is made by the EC, within 67 days after the EMA's recommendation[67]. On the other hand, there is the case of medicinal products for seriously debilitating or life-threatening diseases, including orphan medicines and medicines to fight a public health emergency, for which comprehensive clinical data may not readily be obtained but there is an anticipated positive benefit-risk balance which can be confirmed with post-authorisation clinical data. In this case, the medicinal products can enter the FDA's Accelerate Approval Program[68], in the US, and be granted the EMA's Conditional Marketing Authorisation (CMA), in the EU. After the applicant provides the remaining data, both regulatory entities grant standard approvals. The EMA has one more alternative called Marketing Authorisation under Exceptional Circumstances, which does not have an equivalent in the FDA, for extreme situations where comprehensive safety and efficacy data required are never expected to be obtained, which entails specific monitoring and annual re-assessments[67].

The terminologies and the offices responsible for evaluating the BLA and the MAA are different. 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 (figure 8). Furthermore, in the EU, during the pre-submission stage, in order to determine whether a therapeutic product based on cells, tissues or genes meets the criteria that define ATMPs and what type of ATMP it is, developers can apply for an ATMP classification. After consulting with the European Commission (EC), the EMA delivers its recommendation within 60 days of receiving the request, thus informing the specific dossier requirements and quality guidance to be followed.

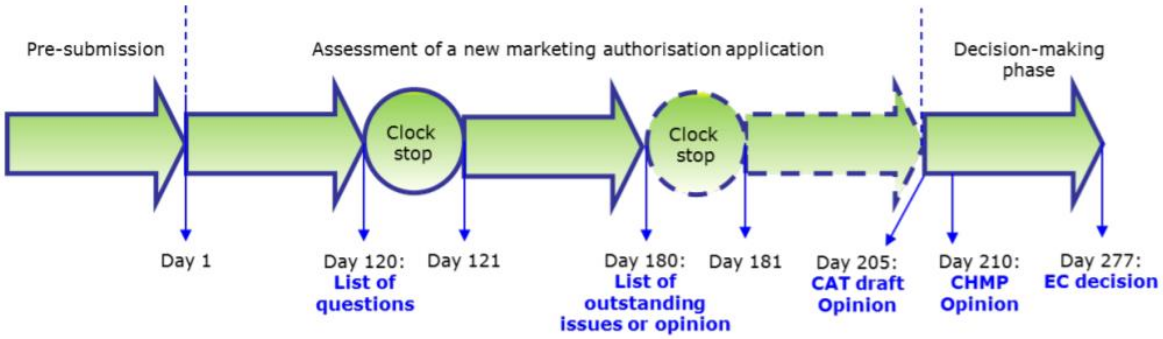


Figure 8 - Overview of the timeline from MAA submission to EC authorisation[69]

This timeline is difficult to recreate for the FDA because the information is scattered throughout many documents, letters and information requests in the approval history of any medicine. Nevertheless, the usual FDA's path for approval is as follows[62](figure 9).

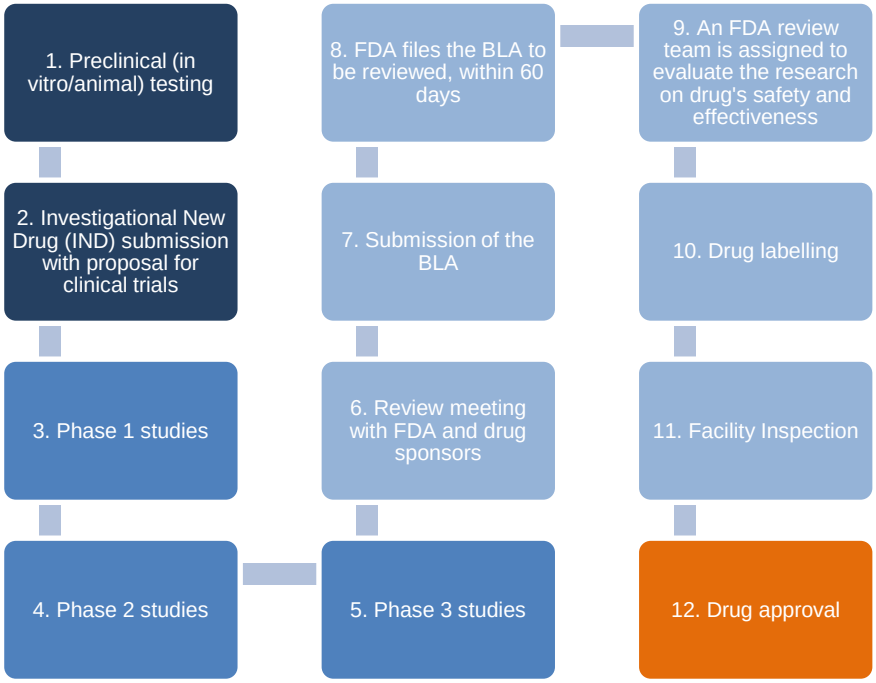


Figure 9 - Schematic representation of FDA's path for approval[62]

It is clear that the EMA and the FDA bare many similarities, namely in the way they have tried to adapt their review and approval process when it comes to advanced medicinal products. Besides providing numerous guidelines for the quality, non-clinical and clinical development of all types of medicinal products, they both provide scientific advice, which not only helps the applicants but also allows to generate extensive discussions and consultations that may contribute to the update of existing guidelines for specific innovative products. Furthermore, both regulatory agencies created expedited development and accelerate assessment programs, which are of upmost importance for the medicinal products that target unmet medical needs or are of major public health interest.

In the end, both regulatory agencies seek to support the medicine development process from an early stage and to offer regulatory mechanisms to help promising new medicines reach patients as early as possible, without compromising their quality, safety, and efficacy.

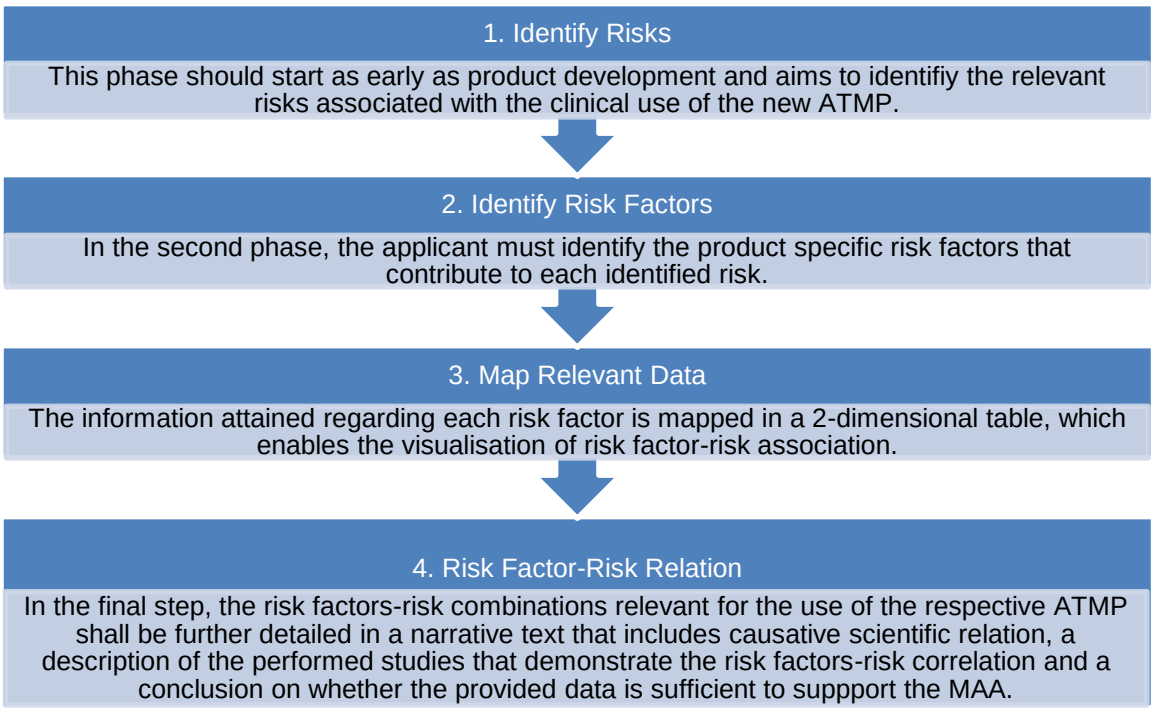
2.4. Risk-based approach for ATMPs

With the constant increase of complexity when it comes to new medicinal products, as well as the growing numbers of MAAs, BLAs and facilities to be analysed by the regulatory entities, it is crucial to have a process that is as optimised as possible. The manufactures want an efficient development and manufacturing so that their product reaches the market as soon as possible. The patients and regulators want safe and high-quality medicinal products, within a timely manner. Everyone involved wishes the development and evaluation processes to be as agile and cost-efficient as possible. With this purpose in mind, the regulators in pharmaceutical industry (both the EMA and the FDA) started to adopt a new methodology called risk-based approach. In the US, the FDA's science and risk-based pharmaceutical quality assessment system is designed to facilitate regulatory flexibility, which also allows for a more efficient drug approval process without compromising product quality and efficacy. This initiative was implemented mainly for the routine surveillance inspection of manufacturing sites and for the monitoring of investigational studies of human medicinal products, not covering the non-clinical development *per se*[70].

In Europe, this methodology has been of particular relevance for advanced and innovative therapies. ATMPs require specific quality, safety, and efficacy demonstrations, which resulted in the introduction of the EMA's Directive 2009/120/EC in late 2009[71]. This directive provides updated definitions of ATMPs and establishes the use of a risk-based approach to adapt the data in the MAA according to the specific risks of the medicinal product, determining the extent of quality, non-clinical and clinical data to be included [67]. The EMA divides ATMPs into four regulatory categories – gene therapy, somatic cell therapy, tissue-engineered therapies, and combined advanced therapies. According to the ATMP Regulation, a product that may fall within the definition of a cell therapy or a tissue-engineered product, as well as a gene therapy medicinal product (GTMP), shall be considered a GTMP, since it is the one that can pose the most safety concerns. According to the Directive 2009/120/EC, gene therapy medicinal product (GTMP) is "*a biological medicinal product that: (a) contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. Gene therapy medicinal products shall not include vaccines against infectious diseases*"[71]. Therefore, CAR T cell products are in fact cell based GTMPs.

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[72]. 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. This methodology is divided in four steps[72]:



Besides the risk profiling for the specific ATMP, the risk-based approach also allows to establish the need for risk minimisation activities and to determine the post market risk management activities to be specified in the pharmacovigilance plan. This is particularly important since CAR T-cells and other GTMP not only distribute extensively in the patient's body but may also persist for a lifetime, hence possible side effects may also occur for an extended period.

3. Methodology

This chapter describes an overview of the research questions that are intended to be answered throughout the multiple studies. It also describes how the relevant documents were attained and analysed. This analysis was conducted with the help of the MAXQDA software, which will be described.

3.1. Research Questions and Data Research

The first part of this thesis focused on reviewing the role of T-cells in immune responses and understanding the benefits and the challenges behind this therapy. For this purpose, an extensive literature review was conducted using the b-on library[73] in which some keywords and expressions used included: *T cell function*, *CAR T Cell Therapy*, *Engineered T-cells*, *Immunotherapy, Drug Development*.

In chapter 2, the approval journey of medicinal products was explored, and the regulatory framework regarding drug development and the existence of regulatory programs in the European Union (EU) and in the United States (US) was compared. The information was attained by navigating the websites of the EMA and the FDA.

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.

To better organise the studies, questions were followed during the multiple assessment steps, taking into consideration previously addressed aspects described in Chapter 2, *Background*, such as i) CAR T-cells definition ii) drawbacks of CAR T-cells iii) stages belonging to the approval of a medicinal product. Furthermore, the non-clinical requirements in guidelines for ATMPs in the US and EU were compared and a case study like exercise comparing CART-cell therapies with other GTMPs has been performed. Studies' questions addressed included:

1. Which CAR T-cells have been approved?

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, which shows the approved ATMPs and which of those are CAR T-cell based. On the other hand, in the EMA's website, it is not possible to conduct a search within a specific pharmaceutical group and so the task of finding approved ATMPs is not easy. For this reason, a literature review was performed to attain the CAR T cell products approved in the EU at the time of analysis.

3.2. 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[74].

In chapter 4, *Results*, a comparison between the non-clinical studies expected for CAR T cell-based products was conducted, with the assistance of MAXQDA.

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*[75]. 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*[76].

3.3. 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 gene therapy medicinal products (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. The use of more medicinal products for this analysis was meant to address more data regarding specific non-clinical studies and understand how the different characteristics of each medicinal product influences the associated non-clinical data package. This analysis was also facilitated by the software MAXQDA.

4. Results

4.1. 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[20], [31], [32]. 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 designations, receiving a standard marketing authorisation[77].

During this chapter, the pathways followed for two of the approved CAR T-cell therapies – Kymriah and Yescarta – were examined.

Kymriah in the EU

To get a better understanding of the steps followed by the EMA after a MAA is submitted, figure 10 illustrates the pathway followed for Kymriah's approval in the EU.

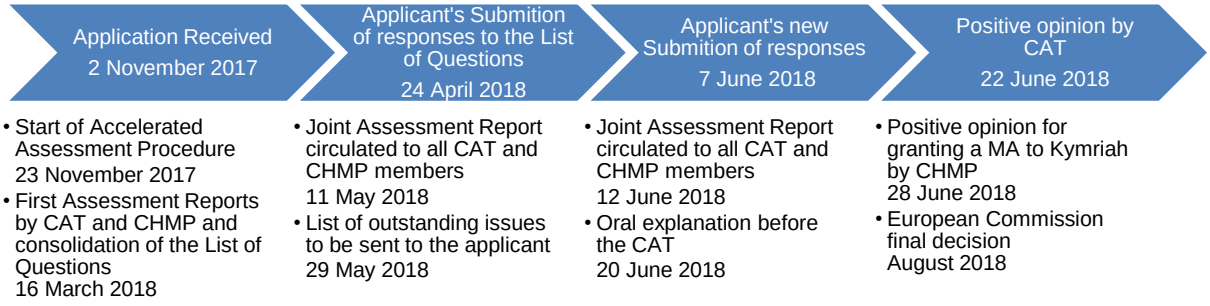


Figure 10 - Pathway followed for Kymriah approval in the EU

After the List of Questions was sent to the applicant in March 2018, there was a clock-stop for the applicant to prepare the responses. During the clock-stop, in March, there was a GMP inspection at the manufacturing site (Novartis Pharmaceuticals Corporation). The first clock-stop ended in April 2018. There was another clock-stop in June, during which the procedure reverted to a standard timetable. Also in June, the CHMP issued a positive opinion for granting the marketing authorisation to Kymriah. The entire process, from the day the EMA received the application to the day the CHMP issued a positive

opinion, took 172 active days. Usually, an Accelerated Assessment takes 150 evaluation days, rather than 210[78], but during the procedure, the CHMP reverted it to a standard timetable. After the EMA's positive opinion, the EC granted the marketing authorisation to Kymriah in August 2018.

Yescarta in the EU and the US

To better understand the comparisons of the programs provided by both regulatory agencies for CAR T-cell products, the comparative timelines of the development and approval by the EMA and the FDA of axicabtagene ciloleucel (commonly known as Yescarta) was recreated and used as example (figure 11).

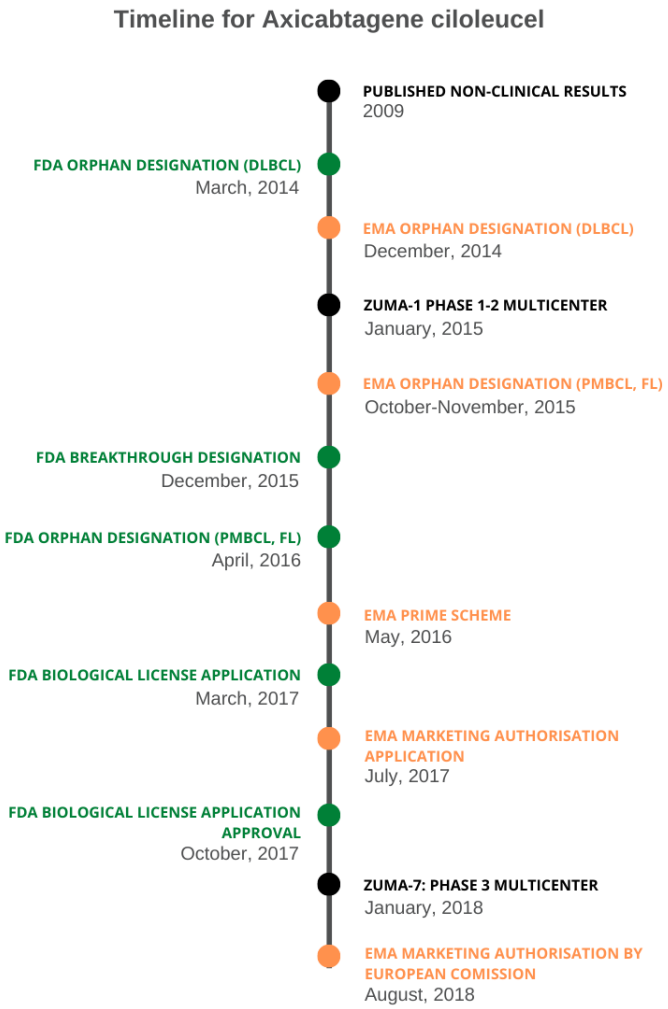


Figure 11 - Timeline comparison for designations and subsequent authorisation of Yescarta in the EU and the US[79]

This comparison showed that the first orphan designation was granted by the FDA, the FDA's Breakthrough Designation was given prior to the PRIME scheme designation, and the BLA was submitted before the MAA. This suggests that Yescarta's applicant initiated the contact with the FDA before establishing contact with the EMA. This may be related to the fact that the FDA offers pre-IND meetings early in the development of a medicinal product, that also allow for a better preparation before the clinical trials begin. In fact, for the other CAR T-cell products, the BLA submissions to the EMA also occurred prior to the MAA submission to the EMA.

Astrazeneca Vaccine

To further demonstrate how all these programs can facilitate the approval of “urgent” medicines, the example of the COVID-19 pandemic has also been scrutinised. In the EU, the EMA created the COVID-19 EMA pandemic Task Force (COVID-ETF) to help the EU Member States and the EC to take “quick and coordinated regulatory action on the development, authorisation and safety monitoring of treatments and vaccines intended for the treatment and prevention of COVID-19”, which involved granting CMAs combined with a rolling review of data during the development of promising therapies and vaccines, to further expedite their evaluation[80]. In the US, the FDA has created a special emergency program called Coronavirus Treatment Acceleration Program (CTAP), responsible for accelerating the development of therapeutics for patients and consumers. Furthermore, the vaccines were given an Emergency Use Authorisation, which allow for a faster distribution in the country[81].

It is important to note that, 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. Notably, in the case of CAR T-cells, as these not only distribute extensively in the patient’s body but may also persist for long periods of time, with possible long term side effects, the pharmacovigilance phase is of utmost importance. Both the EMA and the FDA requested follow-up periods of around two decades for the authorised products[31],[77].

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 as intended and confirm their health benefits.

4.2. 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[20], [31], [32], [77], and are summarised in table 1.

Table 1 - Summary of the approved CAR T cell therapies and respective characteristics and approval dates by the FDA and the EMA

	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

As of February 2021, there were four CAR T cell medicinal products approved in the US and three in the EU, all indicated for haematological cancers. Kymriah and Yescarta were approved in 2017 by the FDA and were the first and second approvals of CAR T-cell immunotherapy, respectively. Both were approved the following year by the EMA. These two approvals represented a big hallmark in cancer therapy and have opened the door for more CAR T cell therapies to be developed and eventually reach the market.

Kymriah™[31]

Tisagenlecleucel (market name Kymriah®) was the first approval of a CAR T-cell immunotherapy, on 30 August 2017, by the FDA, and it represented a major break-through in cancer therapy. It was approved in the European Union (EU) a year later, on 23 August 2018.

It was first approved for paediatric and young adult patients with r/r B-cell ALL after the phase II single-cohort, 25-center, global study ELIANA (NCT02435849), which showed an overall remission rate within 3 months of 81%, an overall survival of 90% at 6 months and 76% at 12 months. Persistence of tisagenlecleucel in the blood was observed for as long as 20 months[82]. The approval of Kymriah for the indication of DLBCL is based on the phase II open-label, multicenter, single-arm study JULIET (NCT02445248), in which it demonstrated a higher response durability compared to the historical control. Kymriah is currently indicated for the treatment of patients up to 25 years of age with r/r B-ALL and adult patients with r/r large B-cell lymphomas after two or more lines of systemic therapies.

Yescarta™[20]

Axicabtagene Ciloleucel (market name Yescarta®) was approved on October 18, 2017, by the FDA and on June 28, 2018, by the EMA, after a single-arm, phase II, multicenter clinical trial where 47% of the participants had a complete response to the treatment. Yescarta is indicated for the treatment of adult patients with r/r DLBCL and primary mediastinal large B-cell lymphoma (PMBCL), after two or more lines of systemic therapy.

Tecartus™[32]

Brexucabtagene autoleucel (market name Tecartus®) was approved by the FDA in July 2020 and received a CMA by the EC in December 2020 for the treatment of adult patients diagnosed with r/r mantle cell lymphoma (MCL). It is the first and only CAR T cell product for adult patients suffering from r/r mantle cell lymphoma (MCL). Tecartus approval is based on the ongoing phase II multicenter study ZUMA-2 for the treatment of r/r MCL conducted at various sites in the US and Europe, which has already generated notable results, as 93% of the infused patients had an effective response and 67% achieved a complete response[83].

Breyanzi™[77]

Lisocabtagene maraleucel (market name Breyanzi®) was approved by the FDA on February 5, 2021, for the treatment of adult patients with r/r large B-cell lymphoma after two or more lines of systemic therapy, including DLBCL not otherwise specified (including DLBCL arising from indolent lymphoma), high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and follicular lymphoma grade 3B.

Breyanzi is the third gene therapy approved by the FDA for certain types of non-Hodgkin lymphoma, including DLBCL. It was approved based on the TRANSCEND NHL 001 trial, which showed a 73% overall response rate and 54% complete response rate.

In the EU, Breyanzi has been granted PRIME designation for r/r DLBCL and its MAA is currently under review by the EMA[84].

To conclude, 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.

4.3. 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.

Both the EMA and the FDA provide guidelines, regulations, and directives to help applicants develop the non-clinical studies and prepare the MAA dossier. Besides these documents, the manufacturers of ATMPs also take advantage of the scientific advice provided by the regulatory entities throughout the development and the manufacturing processes. CAR T-cells are a result of *ex vivo* gene therapy, and, as mentioned before, CAR T-cells are cell-based GTMPs, which means that applicants must take into consideration the guidelines for cell-based medicinal products, GTMPs, medicinal products containing genetically modified cells, specific directives regarding the genetic modification, among others[85].

EMA's recommends a set of guidelines for the non-clinical studies of products containing genetically modified cells. The most recent is the *Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells*[76], which is specific for medicinal products containing allogeneic or autologous somatic cells modified *ex vivo* or *in vitro* with a gene therapy vector prior to administration to the human subject, the *Guideline on non-clinical studies required before first clinical use of gene therapy medicinal products*, the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products*, the *Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors*, and finally the *Guideline on human cell-based medicinal products*[86] and the *Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products*[85].

The recent revision of the *Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells*[76], which was adopted by the CAT and the CHMP in 2020 and already includes specific requirements and recommendations regarding CAR T-cell products, reflects the knowledge that the EMA has gained while evaluating the previously submitted and approved CAR T cell medicinal products and demonstrates the effort made by the EMA to constantly evolve its laws and regulations alongside the advancement of new types of medicinal products.

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*[75] 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*[76] from the EMA (figure 12).

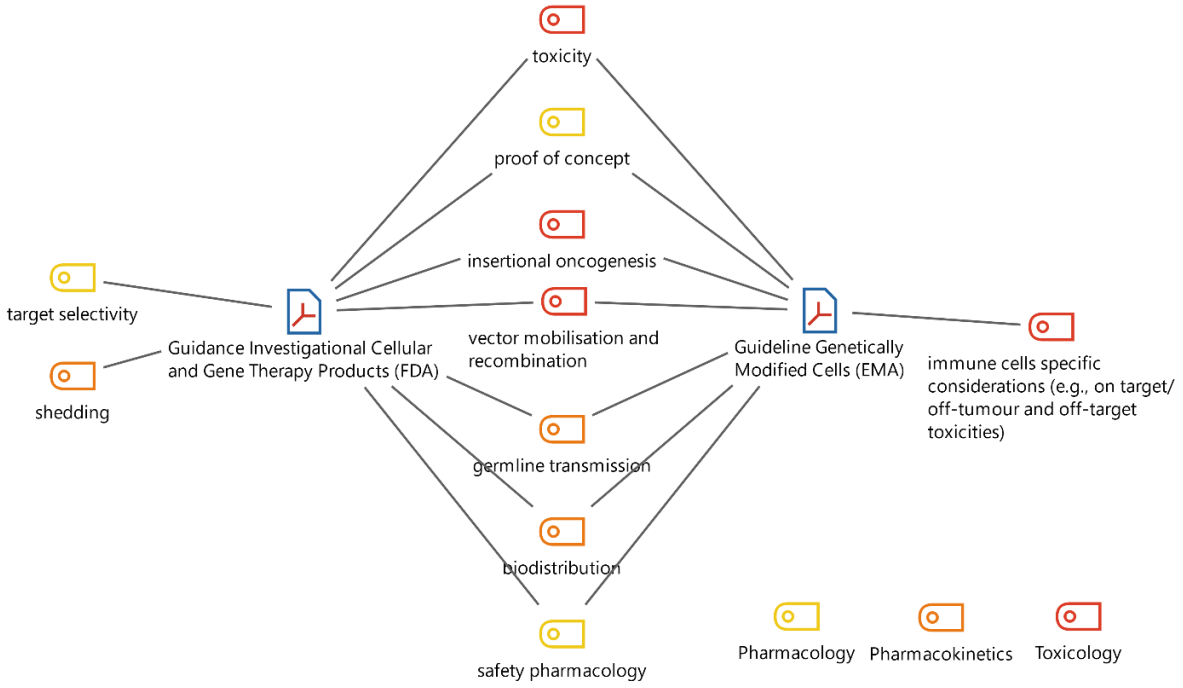


Figure 12 - Scheme obtained from the comparison between the non-clinical studies required in the two guidelines analysed (obtained from MAXQDA)

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 are 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.

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

Following the conclusions presented above on that US and EU requirements for GTMPs are substantially similar, and because the search of documentation regarding non-clinical studies is easier to perform on the EMA's website, the remaining studies were conducted using only documentation from the EMA.

In this chapter, the two facets of CAR T-cells, being both a cell-based product and a GTMP, were explored to get a better understanding of the non-clinical studies required for both types of ATMPs, and to get a better insight on the type of non-clinical studies that exist.

CAR T-cells are a particular type of ATMP as they fit into the cell-based medicinal products as well as into the GTMP designation. As it was mentioned before, the risks associated with GTMP are greater and so, having a risk-based approach in mind, it is understandable that CAR T cell products should be considered GTMPs and evaluated accordingly. Nonetheless, they should also comply with the *Guideline on human cell-based medicinal products*[86]. 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. The types and subtypes of non-clinical studies are the same in both guidelines and are represented in figure 13.

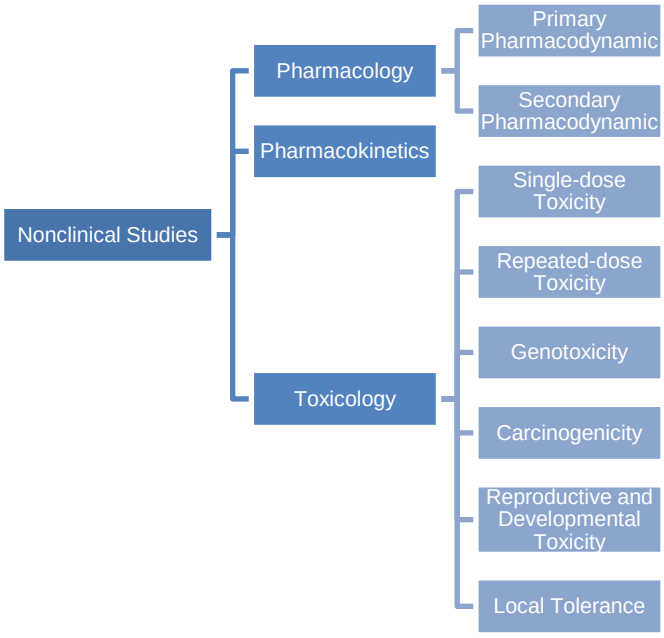


Figure 13 - Non-clinical data package found in the guideline for cell-based products and for GTMPs (obtained using MAXQDA software)

These studies were used as a map in the MAXQDA software to collect the information regarding each study and sub-study. The results are presented in Table 2.

Table 2 - Requirements for each type of non-clinical study mentioned for cell-based products and GTMPs

Non-clinical studies		**Cell-based products**[86]	**GTMP**[85]
Pharmacology	Primary Pharmacodynamic	Demonstrate proof-of-concept; address cell and tissue morphology, proliferation, phenotype, heterogeneity, and differentiation; determine optimal effective dose	Demonstrate proof-of-concept; address level and duration of expression and functional activity; determine optimal effective dose
	Secondary Pharmacodynamic	Assess migration to unintended locations, unintended secretion of proteins and its targets	-
	Safety Pharmacology	Assess central nervous system, cardiac, respiratory, renal or gastrointestinal dysfunctions	Investigate undesirable effects on vital physiological functions; Risk-based approach may be applied
Pharmacokinetics		Standard ADME not relevant; demonstrate tissue distribution, viability, trafficking, growth, phenotype	Standard ADME not relevant; assess distribution, persistence, clearance and mobilisation; address risk of germline transmission and shedding; perform integration studies
Toxicology	Single dose Toxicity	Appropriate post-dose observation period, longer that standard toxicity studies for other biopharmaceuticals; should reflect intended clinical use	Appropriate post-dose observation period, longer that standard toxicity studies for other biopharmaceuticals; should reflect intended clinical use
	Repeated dose Toxicity	Only required if intended clinical use requires multiple dosing	Only required if intended clinical use requires multiple dosing
	Genotoxicity	Not necessary unless direct interaction with DNA	Investigate occurrences of genomic modification, insertional mutagenesis; identify genomic integration sites
	Carcinogenicity	Standard carcinogenicity studies may not be feasible; perform tumorigenesis studies	Standard studies are not required; investigate tumorigenic and oncogenic potential for neoplasm signals, oncogene integration or cell proliferation
	Reproductive and Developmental Toxicity	Case-by-case approach	Investigate germline transmission
	Local Tolerance	Tissue compatibility and tolerance evaluated in toxicity studies	May be needed as part of general toxicity studies

4.4.1. Conclusions on non-clinical recommendations

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.

Interestingly, the overview of both guidelines is similar in suggesting the adaptation of studies according to the nature and risks of the product, requiring justification of the *in vivo* models used for the non-clinical development and/or the use of *in vitro* models in case relevant animal models are not available, and the use of homologous models to overcome the species-specific mismatches. The Guideline for GTMPs offers detailed considerations for selecting the animal model, which should depend on the behaviour of the vector in the animal model (e.g., tissue tropism, selective infection, and possible recombination of the GTMP with endogenous viruses of the animal). This guideline also discusses the use of transgenic models, and of larger animals to better mimic clinical conditions. The chosen animal model(s) may include wild-type, immunocompromised, knock-out, knock-in, humanised or transgenic animals. Notably, the product used in these studies should be representative of the product to be administered to humans, and any deviations should be addressed and justified. Furthermore, it states that the non-clinical development can be designed on the basis of a risk-based approach.

In addition to the general requirements in the *Guideline on human cell-based medicinal products*, GTMPs studies should address toxicity related to the expression of a transgene, risk of insertional mutagenesis, vector mobilisation and recombination aspects related to specific product classes such as immune cells (e.g., CAR and TCR modified T-cells), induced pluripotent stem cells (iPS cells), and *ex vivo* gene edited cells. Furthermore, when the genetically modified cells have a proliferative capacity *in vivo*, such as CAR- and TCR-modified T-cells, clonality and chromosomal integrity may also need to be studied, as well as homogeneity and genetic stability and on-target and off-target effects.

A major difference between the two guidelines is that for GTMPs, an environmental risk assessment (ERA) is usually required, accordingly to the *Guideline on Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products*. The ERA considers the potential adverse effects of the genetically modified organism (GMO)-containing GTMP for the non-patients directly exposed to the GTMP, such as the staff involved in administering the product and patients' family members, as well as the potential adverse effects for animals, plants, micro-organisms, and the environment at large. An ERA should be carried out based on empirically derived data from the quality, preclinical and clinical sections of the dossier, as well as from theoretical assumptions. If unacceptable risks are identified during the ERA, risk reducing measures are defined, and a conclusion on the acceptability of the remaining environmental risk is made. Even though the inclusion of an ERA represents a major difference between the two guidelines, it will not be addressed forward since it is not a non-clinical study *per se*, but more a reflection on non-clinical and clinical data.

Nonetheless, 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.

Recently, the EMA made available a *Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*[87]. The chapter of Non-clinical

Documentation provides the non-clinical data requirements before the first administration of medicinal products in humans and also discusses the points where regulatory flexibility can be applied. Due to the specific characteristics of ATMPs, the majority of non-clinical development is completed before the clinical studies start, hence this draft, in combination with the other two guidelines analysed in this chapter, can be seen as a major source of non-clinical regulatory information for the submission of the MAA. This draft further supports that the extent of the non-clinical data package should be determined on a case-by-case basis, depending on the perceived risks related to the medicinal product and intended clinical use, the availability of animal models, and previous scientific knowledge and clinical experience with similar type of products.

In the end, non-clinical data supporting the safe use of an ATMP (whether genetically modified or cell-based) in humans should provide information for the estimation of the safe and biologically effective dose to be used in clinical trials, support the feasibility of the administration route and the appropriate application procedure, identify safety concerns and target organs for potential toxicity, and identify safety parameters to be followed in the clinical trials[87].

4.5. 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, to facilitate extraction of data from the EPARs rather than using the documents provided by the FDA.

After fully understanding the mechanism behind CAR T cell therapy and the regulatory requisites for cell-based products and for GTMPs, the type of non-clinical development conducted for these medicinal products and whether this development was influenced by a risk-based approach was analysed. Due to the complexity of CAR T cell therapy and the fact that it falls into two different ATMP category, two other approved GTMPs 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 vaccine – 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.

Even though three CAR T cell therapies have been approved by the EMA, only Yescarta and Kymriah were used for the following discussion. Yescarta and Tecartus are products of the same company (Kite Pharma, Inc.), and they have a very similar manufacturing process – both products use the same anti-CD19 CAR construct, vector, final composition, and cryopreservation method. The major changes in manufacturing were aimed at accommodating differences in apheresis material obtained from patients. This also means that the two were subjected to the same non-clinical studies, and so it was decided that the analyses of Tecartus' EPAR would be redundant.

Before further discussion, an overview of the other medicinal products selected for further analysis will be provided.

Astrazeneca vaccine[88]

In 2020, the global pandemic of COVID-19 erupted, changing the world, and taking millions of lives. There was a global effort to find solutions for this pandemic, and academics, researchers and manufacturers, universities and pharmaceutical companies, worked tirelessly to develop, test and manufacture vaccines in order to immunise the population and decrease the effects of the disease.

COVID-19 disease is caused by the infection of severe acute respiratory syndrome coronavirus (SARS-CoV)-2, which has four structural proteins, known as the S (spike), envelope, membrane, and nucleocapsid proteins. The characteristic crown-like spikes seen in coronaviruses are oligomers of the S protein, which bind to receptors on host cells and fuse the viral envelope with host cell membranes. The S protein is considered a relevant antigen for vaccine development because it is the main target of neutralising antibodies, and the vaccination elicits an immune response that prevents infection. There have been outbreaks caused by coronaviruses before, and it is important to notice that, although not commercially available, vaccines have been developed for SARS-CoV and Middle East respiratory syndrome (MERS)-CoV, which allowed to gain valuable data on the coronaviruses and vaccine manufacturing that has been used for the development of vaccines for the SARS-CoV-2[89].

In the case of Astrazeneca vaccine, the viral vector is used to deliver the gene for the coronavirus S protein to the person's cells. When the cells are transduced, the S protein is expressed on the cell membrane. After this, the immune system detects the protein, leading to an immune response with the production of neutralising antibodies as well as the activation of B and T-cells (including memory B and memory T-cells), humoral and cellular immune responses, respectively, directed against the SARS-CoV-2 S protein. The neutralising antibodies and the immune cells will allow a quicker and more effective response in case of a SARS-CoV-2 future infection.

Interestingly, the *Note for Guidance on the Quality, Preclinical and Clinical aspects of gene transfer medicinal products*, published in 2001, served as guidance for both GTMPs and vaccines against infectious diseases. In fact, recombinant technology vaccines like DNA vaccines and viral vector-based vaccines against infectious diseases used to be classified as gene therapies. However, this was updated in the mid-2000s and as a consequence of the new GTMP definition, two other guidelines were developed – the *Guideline for viral vectored vaccines* and the *Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells*, which was already mentioned in this chapter.

Strimvelis[90]

Strimvelis is a cell based GTMP that consists of autologous CD34-expressing haematopoietic stem cells, which are extracted from the patient, purified and transduced (*ex vivo*, as well as the CAR T cell products mentioned before) with a retroviral vector that encodes for the human adenosine deaminase (ADA) cDNA sequence. This treatment is indicated for the treatment of patients with severe combined

immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), for whom no suitable HLA-matched related stem cell donor is available. The transduced cells are expected to engraft into the patient's bone marrow, divide and differentiate in order to repopulate the haematopoietic system with a proportion of cells that express pharmacologically active levels of the ADA enzyme. If the engraftment is successful, its effects are expected to be life-long.

Zolgensma[91]

Zolgensma, which is also a GTMP, is an intravenous infusion of a non-replicating adeno-associated vector (AAV) vector containing the human survival motor neuron gene (SMN1) along with promoters. It is indicated for patients with spinal muscular atrophy (SMA) caused by a mutation in the SMN1 gene, which is usually diagnosed in the first year of life, representing the most severe form of an early childhood disease. The AAV viral vector delivers the SMN1 transgene to cell nuclei where the SMN protein starts being encoded. It is thought that a single infusion of Zolgensma will have a lasting effect throughout the patient's lifetime. This medicinal product is closer to the AstraZeneca vaccine since it also represents the infusion of a virus into the patient.

Viral Vectors

One thing that all the medicinal products mentioned before have in common is the use of viral vectors. Viral vectors are a powerful tool for gene therapy, as they harness the ability of viruses to enter cells to deliver the desired genetic material. Viral vectors have many applications (the genetic modified cells and vaccines against infectious diseases are just two examples), and different types of viral vectors have specific characteristics that make them more adequate for each application. The four main types of viral vectors are: adeno-associated viral (AAV) vectors, adenovirus vectors, lentivirus vectors and gamma(γ)-retrovirus vectors. The choice of vector can be influenced by aspects such as the level of expression of the therapeutic agent, duration of expression, packaging capacity and even the researchers' own experience and preference[92].

After analysing the EPAR of each medicinal product, it was possible to summarise which type of viral vector was used for each one in table 3.

Table 3 - Types of vectors and respective characteristics used for Kymriah, Yescarta, Astrazeneca, Strimvelis and Zolgensma

	Kymriah[31]	Yescarta[20]	Astrazeneca[88]	Strimvelis[90]	Zolgensma[91]
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*

(*) Obtained from the EMA's *Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors*[93]

There is an obvious difference between the vectors used for genetically modified cells and the ones used for Astrazeneca vaccine and Zolgensma, which is the integration ability. Both lentiviral and retroviral vectors have an integration machinery that allows the integration into the host cell chromosomal DNA. In the case of genetically modified cells, the integration of the transgene into the genome of target cells represents the therapeutic goal, as long-term therapy is desired. For both Zolgensma and the COVID-19 vaccine, AAV and adenoviruses vectors are not expected to integrate into the host DNA, as they do not possess integration machinery. However, given the general uncertainties associated with the potential risks of gene therapy, long term monitoring of safety is necessary, mainly related to carcinogenicity in the case of Zolgensma, as this medicine considerably increases the life expectancy of the patients.

Furthermore, the transduction of genetically modified cells is performed *ex vivo* whereas for the other medicinal products, the transduction occurs *in vivo*, representing different levels of control of the genetic modification, which should be reflected in the non-clinical studies of the medicinal products.

The analysis of medicinal products' EPARs enabled the gathering of information related to the non-clinical studies, which were summarised and are presented in the following tables, with a description of the main goals and the methodology used for each. Table 4, 5, 6, 7 and 8 regard Kymriah, Yescarta, Astrazeneca, Strimvelis and Zolgensma, respectively.

Table 4 - Summary of non-clinical studies conducted for Kymriah[31]

Type of non-clinical studies	Subtypes of non-clinical studies	Method and goal of each study	Model
Pharmacology	Primary Pharmacodynamic	Select optimal promoter for expression of the transgene and optimal costimulatory domains in a chromium release T cell killing assay targeting myelogenous leukaemia cell lines	*in vitro*
		Assess CAR+ T cell cytolytic activity against primary B-ALL tumour cells	*in vitro*
		Assess cytokine production of CAR+ T-cells after stimulation with CD19+ tumour cells vs. control cells, using low cytometry-based cytometric bead array	*in vitro*
		Assess proliferation and survival of CAR+ T-cells (without CD19 re-stimulation)	*in vitro*
		Determinate CART-19 specific tumour effects and dose optimisation, using comparison with animals receiving mock-transduced cells or no T-cells as controls	mice
		Determination of threshold of efficacy for CART-19 cells	mice
		Comparison of persistence, anti-B-ALL activity, and effect on survival, using CART-19 T-cells with different costimulatory domains, engineered to express GFP, in immunosuppressed mice	mice
	Secondary Pharmacodynamic	Not conducted	-
	Safety Pharmacology	Not conducted	-
Pharmacokinetics	Biodistribution	Biodistribution of mixture of CART-cells (CAR with vs. without costimulatory domain) in immunosuppressed mice engrafted with human acute B-ALL	mice
	Germline transmission	Not conducted	-
Toxicology	Single-dose	Not conducted	-
	Repeated-dose	Not conducted	-
	Genotoxicity	Lentivirus integration site analysis to test the existence of preferential integration near genes of concern and/or preferential outgrowth of cells harbouring such integration sites during the manufacturing process, using lentivirus insertion site analysis (LISA) and also sonication and shearing-extension primer tag selection (S-EPTS) followed by ligation-mediated PCR (LM-PCR)	*in vitro*
	Carcinogenicity	Not conducted	-
	Reproductive and developmental toxicity	Not conducted	-
	Local tolerance	Not conducted	-
	Other toxicity studies	Assess possible risks of impurities and excipients of the medicinal product	N.A.
Other studies	Tissue cross-reactivity	Cross-reactivity testing of the murine CD19 chimeric antigen receptor single variable fragment equal to the one transduced into tisagenlecleucel cells in a human membrane surface protein array, covering 3550 full human membrane proteins	*In vitro*

Table 5 - Summary of non-clinical studies conducted for Yescarta[20]

Type of non-clinical studies	Subtypes of non-clinical studies	Method and goal of each study	Model
Pharmacology	Primary Pharmacodynamic	Evaluate specificity and potency of the anti-CD19 CAR T-cells by measuring IFN-γ produced by anti-CD19 CART-cells co-cultured with either CD19+ or CD19- target cells, using ELISA	*in vitro*
		Assess characteristics and specificity of the transduced T-cells by comparing phenotype, scFv expression and cytokine induction using a cell line derived from a patient with chronic myeloid leukaemia expressing either CD19 or negative control, using cytotoxic assay	*in vitro*
		Assess product composition by immunophenotypic analysis of surface markers to determine which type of T-cells were being transduced, using flow cytometry	*in vitro*
		Measure 17 different biological factors produced during co-culture of anti-CD19 CAR T-cells and target -cells (either tumour cell line expressing CD19 or negative control cells) to check if CAR products had CD19-dependent activation, using Luminex™	*in vitro*
		Investigate surrogate murine CD19 CAR T-cells for CD19-specific activation by measuring INF-γ release in co-cultures of the anti-murine CD19 CAR T-cells and CD19+ and CD19- target cells	*in vitro*
		Investigate anti-lymphoma effect of the anti-murine CD19 CAR T-cells. Assess CD19 CAR T cell associated survival by administrating the anti-murine CD19 CAR T-cells in both the prophylactic and the therapeutic setting, comparison with control mice	mice
		Investigate influence of total body irradiation (TBI)	mice
	Secondary Pharmacodynamic	Not conducted	-
	Safety Pharmacology	Not conducted	-
Pharmacokinetics	Biodistribution	Assess presence and persistence of the anti-murine CD19 CAR T-cells, evaluated in the syngeneic mouse lymphoma model using flow cytometry analysis	mice
	Germline Transmission	Not conducted	-
Toxicology	Single-dose	No specific single-dose study conducted	-
		Evaluate on-target/off-tumour toxicity of CD19 CAR T*	mice
	Repeated dose	Not conducted	-
	Genotoxicity	No specific genotoxicity study conducted	-
		Address risk of insertional oncogenesis by discussing data gained from clinical experience, and evaluating published literature regarding resistance of mature mouse T-cells to transformation induced by genomic integration of γ-retroviral vectors	-
	Carcinogenicity	Not conducted	-
	Reproductive and developmental toxicity	Not conducted	-
	Local tolerance	Not conducted	-

* study conducted in parallel with the anti-lymphoma effect and the persistence of the anti-murine CD19 CAR T-cells, in pharmacology study

Table 6 - Summary of non-clinical studies conducted for Astrazeneca vaccine[88]

Type of non-clinical studies	Subtypes of non-clinical studies	Method and goal of each study	Model
Pharmacology	Primary Pharmacodynamic	Assess immunogenicity of 1 or 2 doses of testing product by evaluating cytokine response of T-cells using intracellular cytokine staining of splenocytes	mice
		Compare single dose with control using IFN-gamma ELISpot testing in blood mononuclear cells, measure neutralizing antibodies	mice
		Assess immunogenicity and protection against SARS-CoV-2 challenge (with spike protein mutation) - study with 1 dose and 2 doses 4 weeks apart, 28 days before challenge, and post-challenge follow-up of 7 days, different routes - Assess T cell response and pathology with single dose immunisation	non-human primates
		Assess immune response elicited by vaccine and protection after challenge, two complementary studies: measure of viral replication (intranasal vs. intramuscular route) and analyse histopathological features following prime only and prime-boost (4 weeks interval)	ferrets
		Study the humoral and cellular responses response to the vaccine in a large animal model, prime-only vs. prime-boost regimens in small number of animals	pigs
	Secondary Pharmacodynamic	Not conducted	-
	Safety Pharmacology	Specific safety study for cardiovascular and respiratory systems and extrapolation from repeat-dose toxicity study assessing autonomic, neuromuscular, sensorimotor, behavioural parameters and effects on body temperature and pupil	mice
Pharmacokinetics	Biodistribution	Assess the presence of viral vector particles (including assessment of virus replication and disseminated infection): - Ongoing biodistribution following single intramuscular (IM) injection - Study with Same platform vector, IM injection - Study with similar viral vector, intradermally injection	mice
	Shedding	Evaluated in biodistribution studies with urine and faeces analysis	-
	Germline Transmission	Not conducted	-
Toxicology	Single-dose	Not conducted	-
	Repeated-dose	Toxicity assessment of the viral vector studying haematology and plasma chemistry parameters in three supportive studies using different vaccines with the same or a closely related platform and one pivotal study using the vaccine; two doses administered intramuscularly with a 14-day interval	mice
	Genotoxicity	Not conducted	-
	Carcinogenicity	Not conducted	
	Reproductive And Developmental Toxicity	Assessment of reproductive toxicity in supportive studies using the same platform	goat and sheep
		Main DART study is ongoing, final assessment report is demanded post-authorization	mice
	Local Tolerance	Evaluated in repeated-dose studies	mice

Table 7 - Summary of non-clinical studies conducted for Strimvelis[90]

Type of non-clinical studies	Subtypes of non-clinical studies	Method and goal of each study	Model
Pharmacology	Primary Pharmacology	Assess transduction efficiency and vector copy number, using CD34+ cells obtained from umbilical cord blood (UCB) from healthy human donors or BM from ADA-SCID patients transduced with the vector.	*in vitro*
		Assess functional activity with ADA-deficient mice cells and transduced CD34+ cells from BM of ADA-SCID patients, measuring ADA expression by intracellular fluorescence activated cell sorting (FACS) analyses.	*in vitro*
		Assess engraftment and differentiation of transduced CD34+ cells from the bone marrow (BM) of ADA-SCID patients in immunodeficient mice (strain lacking B, T, and NK cells)	mice
		Optimise gene transfer and transduction and assess multilineage differentiation potential	in vitro
		Assess potential of stem/progenitor cells (engraftment and lymphoid reconstitution capacity) in human-mouse chimeras after transduction of culture/transduced CD34+ cells and using CD34+ cells collected from patients after receiving treatment	mice
		Biochemical and functional studies using BM samples collected from ADA/SCID patients under clinical studies harvested before and 10 to 30 months after receiving treatment, using intracellular FACS analysis	*in vitro*
	Secondary Pharmacology	Not conducted	-
	Safety Pharmacology	Not conducted. Transduction of non-target cells addressed in non-clinical biodistribution studies	-
Pharmacokinetics	Biodistribution	Assess engraftment of transduced cells in peripheral blood and lymphoid organs using FACS and VCN analyses in two different groups of pre-conditioning (chemotherapy and irradiation). Definitive study to assess the biodistribution using histopathological analyses on target lymphoid organs and using qPCR in non-target organs such as the brain, heart, kidney, liver, lung, muscle, and gonads.	mice
	Germline transmission	Not conducted. Discussion provided in the Reproductive Toxicology section	-
	Shedding	Not conducted	-
Toxicology	Single-dose	Not conducted. General toxicity endpoints (i.e., clinical observations, body weight, macro and microscopic examination) assessed in definitive biodistribution study	-
	Repeated-dose	Not conducted	-
	Genotoxicity	Not conducted	-
	Carcinogenicity	Study of insertional profile. Tumorigenicity risk assessment based on clinical data and literature on similar vectors due to the inability to achieve long-term engraftment of transduced cells in mice	-
	Reproductive and developmental toxicity	Not conducted. Discussion about the risk of germline transmission	-
	Local tolerance	Not conducted	-

Table 8 - Summary of non-clinical studies conducted for Zolgensma[91]

Type of non-clinical studies	Subtypes of non-clinical studies	Method and goal of each study	Model
Pharmacology	Primary Pharmacology	Assess distribution and subsequent transgene expression in neonatal and adult mice, using a AAV9 vector similar containing a sequence for GFP protein. Assess timing of administration using AAV9-SMN1.	mice
		Analyse expression of human SMN protein, body weight, survival, and motor functions after systemic administration of the medicinal product. Compare dose and efficacy of intracerebroventricular (ICV) and IV administration. Analysis of IV vs IT administration was lacking but considered important	mice
		Assess distribution of the vector with GFP and subsequent transgene expression after dosing via intrathecal or via intracisternal, using histology and immunohistochemistry evaluation. Analyse dose and efficacy of the medicinal product via IT in a spinal muscular atrophy (SMA) model.	piglet
		Assess distribution of the vector with GFP and subsequent transgene expression after dosing via IV and via IT.	monkey
	Secondary Pharmacology	Assess efficacy of the product to reverse disease-related cardiac deficits	mice
	Safety Pharmacology	Not conducted	-
Pharmacokinetics	Biodistribution	Assess biodistribution and persistence of Zolgensma vector DNA as well as RNA expression of the hSMN transgene, by collecting tissues for qPCR distribution and RNA expression analyses, in the context of a preliminary 6-months IV safety and biodistribution study, with non-validated analytical methods.	mice
		Assess safety and biodistribution studies in two 3-months IV, characterized by validated analytical methods, by collecting tissues for ddPCR and RNA expression analyses.	mice
	Germline transmission	Not conducted	-
	Shedding	Not conducted	-
Toxicology	Single-dose	Test safety and biodistribution, through clinical observations, changes in blood parameters and pathological abnormalities, when using intravenous route and different doses in 12 and 24* weeks GLP compliant studies. Define maximum tolerated dose	mice
		Evaluate toxicity when using intravenous route using a low dose of an unknown batch*	NHP
		Determine safety and biodistribution when using intracerebroventricular route and single dose in an exploratory study*	mice
		Determine safety and biodistribution when using intrathecal route and single dose in an exploratory study*	NHP
	Repeated-dose	Not conducted	-
	Genotoxicity	Not conducted	-
	Carcinogenicty	Not conducted	-
	Reproductive and developmental toxicity	Not conducted. Present in detail discussion of existing literature data on inadvertent germline transmission of AAV vectors and potential interference of SMN overexpression with early embryonic development	-
	Local tolerance	Not conducted	-

*performed with research batch (different manufacturing process), hence these studies were not regarded of additional value for the estimation of the clinical risks for Zolgensma

4.6. Pharmacology

4.6.1. Primary Pharmacodynamic

All EPAR documents demonstrated the importance of having proof-of-concept studies in the non-clinical development. The goal of these studies is similar for all medicinal products – understand the effects of their administration and whether they are aligned with the intended therapeutic outcome. However, when comparing the studies conducted for Yescarta, Kymriah and Strimvelis with the ones for AstraZeneca and Zolgensma, the difference between the chosen non-clinical models is evident. Genetically modified cells EPARs report numerous *in vitro* studies, whereas the other two EPARs report the use of animal models only. This shows the different adaptations of pharmacodynamic studies according to the type and intended therapy of the medicinal product.

For Yescarta and Kymriah, the *in vitro* studies were used to determine the best costimulatory domain, assess the specific activity of CAR T-cells against its target antigen CD19, measure cytolytic activity and cytokine production in the presence of CD19+ tumour cells, and characterise the final product regarding the types of T lymphocytes being transduced (the Kymriah applicant did not conduct these phenotyping studies but the EMA considered there was enough information gathered through clinical experience). These *in vitro* studies are of upmost importance for CAR T cell therapies as *in vivo* models still provide very limited information and extrapolation to humans. Furthermore, even though the same animal was used for the *in vivo* studies of Yescarta and Kymriah, the models were not the same. The *in vivo* pharmacology studies of Kymriah were conducted in immunocompromised mice transplanted with human CD19+ tumour cells to assess the behaviour of the CAR T-cells. The *in vivo* studies of Yescarta, on the other hand, used a syngeneic mouse lymphoma model (i.e., immunocompetent mice) and tested the proof-of-concept using murine surrogate CD19 CAR T-cells. In this model, both the lymphoma cells and the transduced T-cells are of murine origin, and so the surrogate CAR construct was built with a scFv that recognises murine CD19 (the actual CAR construct of Yescarta only recognises human CD19), and no human derivatives were used. Both models have their own benefits and limitations, which will be discussed further. Notably, the mice models used for primary pharmacodynamic studies were also used for other *in vivo* non-clinical studies, for both Kymriah and Yescarta.

The pharmacodynamic studies conducted for Strimvelis were designed to assess transduction efficiency, vector copy number, and functional activity (i.e., ADA expression) in the *in vitro* models, and engraftment, transduction, and differentiation in the *in vivo* model, which was also the immunodeficient mice.

AstraZeneca, on the other hand, was only studied in animal models. The vaccine is not target-specific, and its main goal is to produce an immune response, which will then offer protection in the case of an infection. Therefore, the pharmacodynamic studies for Astrazeneca are mainly focused on evaluating the immunogenicity, which included the measuring of antibody titers and cytokine production. In order to assess the immunogenicity and compare the protection using one or two doses of the vaccine, various animal models (i.e., mice, non-human primates, ferrets and pigs) were used, as this cannot be assessed

in vitro. Notably, during the studies conducted for non-human primates, unexpected finding of viral RNA in tissues of the gastrointestinal tract was found at 7 days post-challenge in immunised animals, which was not further addressed. Furthermore, regarding the study in ferrets, the EMA raised some questions about the limited assessment of humoral and cellular immune responses and recommended the applicant to submit further data on antibody subtypes, Th1/2 response (the lack of a balanced ration can lead inappropriate immune responses), T cell subtyping and determination of neutralising antibodies after vaccination and challenge.

In the case of Zolgensma, which is a therapeutic vector, the focus was on vector distribution, transgene and protein expression, while comparing different administration routes in various animal models.

4.6.2. Secondary Pharmacodynamic

CAR T cell products, AstraZeneca and Strimvelis were not subjected to these studies because they are not feasible in animals or relevant due to the nature of the products.

In the case of Zolgensma, it is mentioned the study of the medicinal product efficacy to reverse disease-related cardiac deficits. In SMA, the loss of motor neurons leads to secondary effects on muscle strength and function, leading to progressive loss of muscle control, strength and function, swallowing, breathing and, ultimately, death. Although the primary pathology of SMA is neurodegeneration at the level of the spinal motor neuron, some clinical reports indicate the involvement of other organs such as the heart. Therefore, it is reasonable to evaluate the effects of the medicinal product on this organ. However, the EPAR asserts that the correction of cardiac dysfunction in mice may be due to the transduction of the heart muscle cells directly (i.e., secondary pharmacodynamic effect) or it can be resultant of the transduction of neurons that stimulate heart contraction (i.e., primary pharmacodynamic effect), which could be a topic of further discussion.

4.6.3. Safety pharmacology

Applicants of CAR T cell therapies did not conduct any safety pharmacology studies, which was considered acceptable based on the limitations of the available animal models. Regarding the other two GTMPs, safety pharmacology studies were not conducted for neither of them. The discussion presented for Strimvelis was that the ADA protein expression and enzymatic activity following transduction is expected to be below or equal to physiological levels, therefore adverse functional consequences are not expected even if the transgene is inadvertently expressed in non-targeT-cells (e.g., heart). Nevertheless, the applicant addressed the transduction of non-targeT-cells in the non-clinical biodistribution studies. In the case of Zolgensma, the goal is to have the expression of the SMN1 protein in patients that have a deficiency, and so it is also not expected to have hazardous effects in vital physiological functions.

On the other hand, AstraZeneca's EPAR indicated that the applicants conducted a safety study for cardiovascular and respiratory systems and also extrapolated data from repeated-dose toxicity study

assessing autonomic, neuromuscular, sensorimotor and behavioural parameters and effects on body temperature.

4.7. Pharmacokinetics

Biodistribution studies

The biodistribution studies of CAR T-cells focused on *in vivo* persistence, expansion, and survival of these cells in the chosen animal models. Once again, Kymriah was tested in an immunocompromised mice engrafted with human leukaemia cells. In the case of Yescarta, it was not the Yescarta product *per se*, but rather its murine surrogate, that was evaluated in the syngeneic mouse lymphoma model.

When it comes to AstraZeneca, since it is replication-incompetent in human cells, no further infection or spread of the virus is expected after the initial infection of the cells upon viral entry, and the biodistribution studies are vital to confirm this. The applicant presented two previously conducted biodistribution studies, one using the same platform vector with a different insert and other using a different adenovirus with similar infectivity. The identical platform study evaluated the biodistribution in the blood and different organs, but left out the CNS, peripheral nerves, and bone marrow, and some methods used were not validated. The related vector study also showed some veracity concerns and lack of validation, so the relevance of the collected results was deemed limited and triggered the need for a new specific biodistribution study which is ongoing and will include the assessment of the aforementioned tissues that were left out, in addition to faeces samples. The data of this ongoing study must be submitted post-authorisation.

The biodistribution studies of Strimvelis – pilot and definitive study – focused on the engraftment of transduced cells and the assessment of immune reconstitution. The presence of human cells was assessed in both target lymphoid organs and non-target organs (e.g., brain, heart, kidney, liver, lungs, muscle, and gonads). In the case of Zolgensma, the focus was on the distribution and persistence of the vector DNA as well as RNA expression of the transgene, both in the preliminary biodistribution study and in two other biodistribution studies. In the latter, the tested tissues were those involves in the pathology of the disease or expected to be highly transduced due to vector tropism. Furthermore, two clinical autopsy reports that assessed a wider range of tissues were presented. The applicant also discussed similarities and differences in extrapolation between mouse and human.

Germline Transmission

The risk of germline transmission associated with the administration of genetically modified human cells is considered low and difficult to address in non-clinical germline transmission studies, therefore, these studies are not recommended, unless otherwise justified[93]. This explains the lack of these studies for Kymriah, Yescarta and Strimvelis. However, the treatment with Strimvelis entails a pre-conditioning treatment with busulfan, which has a known gonadotoxic effects in humans and animals that was confirmed in the biodistribution study in mice.

In the case of Zolgensma, the low but persistent levels of vector DNA observed in gonads should have triggered further non-clinical analyses in accordance with the *Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors*. The lack of these studies was justified with the discussion of existing literature data on inadvertent germline transmission of AAV vectors.

For Astrazeneca vaccine, studies that assess the potential risk of germline transmission were not carried out. This was justified due to inexistent reported cases of germline transmission of replication-deficient adenovirus in animal models or humans.

4.7.1. Shedding

For CAR T-cells and for Strimvelis, the viral vector is introduced into the T-cells *ex vivo*, and so it is not administered to the patient directly. This means that there is no risk of shedding after the therapy, therefore these studies are not appropriate. For Astrazeneca, there was a shedding assessment in urine and faeces using a similar vector with different insert and it will also be addressed in the ongoing biodistribution study.

For Zolgensma, it would be expected to have a shedding assessment of the vector, however, despite being considered an integral part of biodistribution studies, this was not conducted. The applicant provided a human shedding data of AAV9 (based on literature studies) in the clinical section of the EPAR, which was considered sufficient.

4.8. Toxicology

4.8.1. Toxicity study

Single dose toxicity

There is no relevant animal model for addressing toxicity of engineered human T-cells, and so the lack of single-dose toxicity studies is acceptable for Kymriah and Yescarta. Notably, the Yescarta applicant presented an on-target/off-tumour toxicity study of the CD19 CAR T-cells in the syngeneic mouse lymphoma model, which has been evaluated during the pharmacology study in parallel with the anti-lymphoma effect and the persistence of the anti-murine CD19 CAR T-cells. This *in vivo* study was not conducted for Kymriah, possibly due to the chosen murine model.

In the case of Strimvelis, no single dose toxicity study was conducted but general toxicity endpoints were assessed during the definitive biodistribution study. Zolgensma, on the other hand, was subjected to multiple studies which generated data with limited relevance and validity but allowed to define the maximum tolerated dose.

In the case of AstraZeneca, because it is meant to be administered in two doses, the single dose toxicity study is not relevant.

Repeat dose toxicity

The lack of repeat-dose toxicity studies is not applicable when the ATMP is administered as a single infusion, which is the case of Yescarta, Kymriah, Strimvelis and Zolgensma.

In the case of AstraZeneca, once again, the applicant submitted information of support studies that used the same platform or the same vector with a different insert, conducted in mice. Besides these studies, the applicant also conducted another study using the actual AstraZeneca product that measured changes in body temperature and haematology and plasma chemistry parameters. The use of only one animal model in these studies is in accordance with the World Health Organisation Guideline for vaccines[94].

4.8.2. Genotoxicity

Assessment of insertional mutagenesis risk was not considered for products using non-integrating vectors, such as AstraZeneca (adenoviral vector) and Zolgensma (AAV vector). The risk is higher in products using integrating vectors, such as Yescarta and Strimvelis (retroviral vector) and Kymriah (lentiviral).

Yescarta applicant addressd the risk of insertional mutagenesis/oncogenesis in the *Genotoxicity* section with detailed evaluation of published literature regarding resistance of mature mouse T-cells to transformation induced by genomic integration of γ-retroviral vectors. Kymriah's applicant provided a lentivirus integration site analysis to test the existence of preferential integration near genes of concern and/or preferential outgrowth of cells harbouring such integration sites during the manufacturing process. In the case of Strimvelis, conventional genotoxicity assays are deemed inappropriate to detect insertional events and no studies have been conducted, but the insertional mutagenesis/oncogenesis was addressed in the *Carcinogenicity* section. This can be explained given that insertional mutagenesis (i.e., alteration in the genome) is considered a basis for carcinogenicity (i.e., the formation of tumours).

4.8.3. Carcinogenicity / Tumorigenicity

These studies were not conducted for any of the medicinal products. For both CAR T cell products, their omission was justified based on literature and clinical data (in the case of Yescarta) and the lack of appropriate animal models (in the case of both Yescarta and Kymriah).

In the case of Strimvelis, the applicant received scientific advice recommending that in case the optimal conditions for a full tumorgenicity study could not be determined, the carcinogenicity assessment could be based on clinical data and literature on similar vectors. Therefore, since it was not possible to demonstrate long-term engraftment of transduced cells in mice, the applicant presented the suggested alternative.

Once again, in the case of Zolgensma, these studies were not presented, nor their omission justified. However, no cases of insertional oncogenesis have been reported for AAV vectors, so far, hence the tumorigenic potential of these medicinal product is regarded to be very limited.

In the case of Astrazeneca vaccine, the applicant stated that studies evaluating genotoxicity and carcinogenicity are normally not required for viral vaccines, hence these were not conducted.

4.8.4. Reproductive and developmental toxicity

No reproductive or developmental toxicity studies were conducted with CAR T cell products nor with Strimvelis. For the latter, the discussion about the risk of germline transmission was included in this section. Yescarta's assessment report stated that the lack of studies to evaluate the effects of Yescarta on fertility, reproduction, and development was considered acceptable based on the type of product, the expression pattern of the target antigen and the lack of a relevant animal model. However, Yescarta is not recommended for pregnant women or for women of childbearing potential not using contraception since the potential to cause foetal toxicity is not documented.

In case of Zolgensma, these studies were not conducted but a detailed discussion was presented. On one hand, the risk for transfer of a non-integrative, non-replicative vector to offspring via spermatocytes is low, and, on the other hand, the modified AAV vector cannot in theory multiply itself. Therefore, for male patients, in case spermatocytes or its progenitors are transduced with the medicinal product, and since Zolgensma is to be administered to children usually under 6 months, it is anticipated that the vector will be diluted out. For female patients, oocytes are all present before birth, hence transduction of oocytes could potentially lead to transfer of the vector to offspring, but it is expected to dilute out in the developing embryo. The applicant further discussed the potential interference of SMN overexpression with early embryonic development.

For AstraZeneca, the reproductive toxicity was assessed in supportive studies using the same vector platform and a main (developmental and reproductive test) DART study was initiated, the final assessment report must be submitted post-authorization.

4.8.5. Local tolerance

Neither Yescarta nor Kymriah applicants provided local tolerance studies as it is not appropriate or this type of therapy. In the case of Strimvelis, local tolerance studies were deemed unnecessary by the applicant and the EMA. Zolgensma's EPAR also did not mention any local tolerance studies.

In the case of AstraZeneca, the only non-GTMP medicinal product, the local tolerance study was evaluated in repeated-dose studies, which is in accordance with the WHO Guideline for vaccines[94].

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

From the analysis conducted with all medicinal products' assessment reports, it was possible to observe some differences and similarities, which is aligned with the fact that all these products use the same technology of viral vectors but with different types of vectors and for different therapeutic purposes. Furthermore, 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. In addition, the extent of non-clinical studies was also dependent on the availability and relevancy of *in vivo* models.

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. In this regard, the following discussion will approach the main risks and risk factors of the medicinal products mentioned above. The medicinal products 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.

4.9.1. 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.

Treatment failure

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 sFv affinity with CD19 as well as the persistence and expansion of the CAR T-cells, and associated survival. The specificity of CAR T-cells against their target was thoroughly evaluated both *in vitro* and *in vivo*. Furthermore, Kymriah's applicant also provided a tissue cross-reactivity study between the murine-origin scFv CAR and multiple human proteins to guarantee the specificity against the CD19. In the case of Strimvelis, the engraftment and differentiation capacity of the CD34+ cells are essential to ensure that the therapeutic desired outcome is reached. When using this medicinal product, the patient can have an unsuccessful response to the gene therapy due to anti-ADA antibodies from previous therapy and autoimmunity, however, due to patient-specificity, this can only be assessed during clinical trials, and so no non-clinical study was conducted to address it.

Toxicity/Immunotoxicity

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 ADA enzyme at or below physiological levels, hence toxicity studies related to the effects of the enzyme are not relevant. However, as it was mentioned above, 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.

Tumour Formation

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, which was done for both CAR T-cells. 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.

Another risk factor related to tumour formation is the potential for vector replication, however, all medicinal products use replication defective or incompetent viral vectors, so this risk is minimised. In any case, the replication potential was tested during manufacturing and addressed in the ERA.

4.9.2. Non-integrating vector and *in vivo* genetic modification

Both Zolgensma and Astrazeneca vaccine 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. In fact, there are clear distinctions between these two products. Zolgensma is administered intravenously and contains the SMN1 gene, which encodes the SMN protein, essential for the normal functioning of nerves that control muscle movements. The SMN1 gene is defective in SMA type 1 patients, but Zolgensma delivers a functional copy of it, correcting the protein production and restoring nerve function. On the other hand,

Astrazeneca vaccine is administered intramuscularly, and delivers the gene that encodes the SARS-CoV-2 spike protein, which will be transduced by the person's cells, leading to the production of antibodies and the activation of T-cells against this protein, preparing the immune system in case of a future infection. Both vectors deliver a protein to be produced by the human cells, but for Zolgensma, the transduction of motor neurons is key to treatment success so the efficient distribution of the vector to these neurons is a major focal point, whereas for Astrazeneca vaccine, there are no specific target-cells. In the case of Zolgensma, the production of SMN protein is the desired outcome, as this corrects the protein deficiency in SMA patients and thereby restores nerve function. In the case of the vaccine, the production of the protein still needs to cause an immune response that will then produce the necessary defences in case of future infections. 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.

Unwanted Immunogenicity

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 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 AAV9 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 AAV9-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 against platelet-factor 4) was observed in patients receiving this vaccine[95]. 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.

Inadvertent Germline Transduction

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.

In the case of Zolgensma, there was low but persistent levels of vector DNA observed in gonads of monkeys, which should have triggered further non-clinical analyses in accordance with the *Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors*[93]. To justify the lack of these studies, the applicant thoroughly discussed existing literature data on inadvertent germline transmission of AAV vectors, and concluded that despite being distributed into gonadal tissue, the risk of transducing these cells and resulting in germline transmission is low.

In Astrazeneca assessment report, the applicant also explained the absence of germline studies due to the type of the vector. Instead, the reproductive toxicity was a focus point, and the applicant was required to deliver a DART study to complete the already submitted information. Following the conditional marketing authorisation, the DART study revealed that the vaccine "*elicited detectable anti-SARS-CoV-2 S-glycoprotein maternal antibodies being transferred to the foetuses and pups, indicating placental and lactational transfer, respectively*", however, these animal studies did not indicate harmful effects with respect to pregnancy, foetal development, birth or post-natal development. Once again, there was no mention to the antibodies against the vector itself. Although the DART study findings do not mention it, if the vector distributes to the gonads, then a germline transmission study could have been relevant, according to the *Guideline on non-clinical testing for inadvertent germline transmission of gene transfer vectors*[93].

4.9.3. 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* models 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[87].

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.

Both Strimvelis and Kymriah used immunodeficient mice during non-clinical studies. Interestingly, even though both CAR T cell therapies have the same route of administration and a similar therapeutic effect, they were tested in two different models. The applicant of Kymriah tested the human-origin CAR T-cells against human tumour cells, using the "conventional" model of immunosuppressed rodents. This model has two main limitations: the human cells may cause a xenogeneic immune response that is unrelated to the effect of CAR T-cells, and it does not reflect the intricate interactions of the CAR T-cells with other components of the immune system since the mice are immunocompromised. On the other hand, the applicant of Yescarta chose a homologous model to test not the product itself but a similar product using murine cells. This allows the use of an immunocompetent mice to test the immune system response, which is particularly meaningful in this type of therapy, even though the data obtained is conceptual, since the actual medicinal product (that uses human cells) is not being tested. However, this model comes with constraints of its own, as some factors may differ between surrogate murine and human CAR T-cells, such as the T cell characteristics that are innate to each species. The immune system of the mice is not the same as the human, and so the extrapolation of the non-clinical pharmacology data to human is still limited. Both models have their own benefits and limitations, the surrogate model may be better at addressing two very important characteristics of the CAR T-cells, their persistence and the possible on-target/off-tumour effects. The acceptance of *in vivo* studies that do not test the actual candidate for clinical trials was justified on the fact that the "conventional" model does not offer a better data extrapolation along with the fact that Yescarta was tested in multiple *in vitro* studies. Nonetheless, this shows that past studies can influence the type of models that are considered acceptable. In fact, according to the *Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*, the use of homologous animal models is encouraged when they are expected to provide more reliable data than non-homologous models[87]. This flexibility of the design of non-clinical studies is tightly related to a risk-based approach, not only regarding the selection of animal models but also in the planning of necessary study packages depending on the type of previous knowledge of a specific medicinal product.

Ultimately, there is no single optimal preclinical model for CAR T therapy, but developments in breeding transgenic mouse strains, improvements in the humanisation of murine immune systems, and the combination of multiple animal models may provide more information of different aspects regarding CAR T-cells. The evolution and refinement of preclinical models will lead to improved prediction of CAR T safety and efficacy in the clinic[21]. Notably, the welfare of animals used in research is of upmost importance for the regulators and a cornerstone for future research, and there is a continued effort to replace, reduce and refine the way experiments are carried out. 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[96], [97]. 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 vaccine 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.

4.9.4. Analysis of shared non-clinical strategies

Even though the risks associate with each medicinal product and the chosen animal models were different, all these medicinal products had overlapping study goals during non-clinical development. In order to better perform this comparison, and taking advantage of the regulatory flexibility subsequent to the use of a risk-based approach during non-clinical development, the *Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*[87] was considered pertinent to be analysed alongside the conclusions gathered during previously discussed study phases. This draft highlights the specific risks and risk factors that should be addressed prior to human administration for a safer clinical development.

It is important to emphasize once more that Astrazeneca vaccine does not fall within the scope of ATMPs, however, the fact that its mode of action requires a genetic modification prior to the common vaccine immune response, and the fact that this is attained using viral vectors, brings this vaccine closer to the other advanced therapies mentioned in this work.

Pharmacodynamic Studies

Data demonstrating proof of concept was collected for all medicinal products, which allowed to support the therapeutic rationale and the safety of the products. The previously mentioned guideline defends the use of *in vitro* models to complement animal models, which was followed by the products using genetically modified cells, as the relevancy of animal models is limited. Both Zolgensma and the vaccine took advantage of larger animal models to further study their expected outcomes. For all the products, the transduction and subsequent expression of transgene product was evaluated, and genome integration was discussed, with genome integration studies being performed for Kymriah and Strimvelis.

Both secondary pharmacodynamic and safety pharmacology studies were considered on a case-by-case basis.

Pharmacokinetic Studies

These studies allowed to confirm the results obtained in pharmacodynamic studies and further evaluate biodistribution. For all the analysed medicinal products, the standard Absorption, Distribution, Metabolism and Excretion (ADME) pharmacokinetic studies were not considered relevant. These data also provided information on the persistence, duration of effect, and target cells/organs, which, according to the *Draft Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials*[87] is essential to support the design and duration of clinical studies. All the evaluated medicinal products conducted these studies in mice.

Toxicology Studies

Once again, all the evaluated products were subjected to toxicology studies. For some products, the assessment of toxicology endpoints was performed during other non-clinical studies, e.g., pharmacology and biodistribution studies. When it comes to genotoxicity, special attention was given to insertional mutagenesis evaluation for the *ex vivo* genetically modified cells – Kymriah, Yescarta and Strimvelis. On the other hand, reproductive and developmental toxicity was more thoroughly discussed for the Astrazeneca vaccine and Zolgensma. Notably, for some medicinal products, the applicant only provided a discussion or literature review and not data obtained from studies. This is accordingly to the Draft Guideline, which defends that the extent of non-clinical data is dependent on the perceived risks of the product and previous scientific knowledge [87].

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.

These new types of advanced medicines, with a small target population but a high associated cost, are an important point of discussion for healthcare systems, which must ensure these products reach the patients that need them. The high price associated with CAR T-cell therapies may be justified since they attend conditions with no other alternative treatment and also with the investment made by pharmaceutical companies during the development of the products, which is fair since it takes many years and heavy investment for a medicinal product to reach the market. It is not uncommon, however, that the governments help to fund clinical trials and, as it was analysed, the regulatory entities offer fee reductions in order to help this development. In fact, the approval journey of CAR T cell-based therapies benefited from many regulatory programs from both the EMA and the FDA, which allowed for more intense scientific advice and protocol assistance, as well as financially supportive measures.

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.

In chapter 4, during the comparison of non-clinical requirements for CAR T-cells in the EU and the US, it was concluded that, even though the requirements for both regulators were similar, the assessment of non-clinical studies performed for the medicinal products were easily enumerated and described in the EMA's assessment reports but were not readily available in the FDA's website. For this reason, the research steps that followed were based solely on the documentation provided by the EMA.

The following study performed compared two guidelines, namely the *Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products*[85] and the *Guideline on human cell-based medicinal products*[86], which allowed to understand the different requirements for both types of ATMPs and how complex it is to assess CAR T cell products that belong to both categories. Furthermore,

the influence of a risk-based approach, even implicit, was noticeable as the study requirements greatly depend on the risks usually associated with cell-based products and with genetically modified products.

The CAR T-cell therapies' non-clinical package was then compared to other GTMPs and to the COVID-19 vaccine Astrazeneca. Even though gene therapy products do not include vaccines against infectious diseases, the Astrazeneca 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.

When it comes to animal model selection, it is important to follow the 3Rs principles: replace the use of animals with alternative techniques or avoid the use of animals altogether in case they do not provide meaningful supportive data; reduce the number of animals used to a minimum, to obtain information from fewer animals or more information from the same number of animals; and refine the way experiments are carried out, to reduce animal suffering as much as possible. Continuing to search for new models that do not involve the use of animals such as cell- and tissue-based models, including organoids and microfluidics, is essential to facilitate future non-clinical development.

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. Moving forward, it could be helpful to have a uniformization of documents in the two analysed regulatory entities. In the case of the EMA, the number of available guidelines applicable for each product is quite high and so there is a lot of information to consider, which makes it understandable that early scientific guidance can be decisive for the applicants to conduct a successful development and entrance to the market. 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.

6. References

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