TÉCNICO LISBOA

First-in-Human for Advanced Therapy Medicinal Products

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Abstract

Remarkable efforts have been made in the development of novel medicinal products, allowing to shift from a “one size fits all” healthcare approach to a personalized medicine. Advanced therapy medicinal products (ATMPs), based on cells, tissues or genes, offer innovative solutions to unmet medical needs and life-threatening conditions, representing a groundbreaking pharmaceutical field that shows great value but face some complex challenges. Due to ATMPs complexity and unique characteristics, regulatory framework must be tailored on a case-by-case basis, to efficiently evaluate these medicinal products for further market entrance. The present master thesis aims to discuss preclinical requirements, mandatory for conducting first-in-human studies of ATMPs, with a focus on cell-based medicinal products and using NVivo software for a more efficient analysis. Firstly, scientific guidelines amending non-clinical studies for small molecules, biotechnology pharmaceuticals, cell-based and gene therapy medicinal products were compared. Secondly, six different ATMPs case studies were analysed, assessing similarities and dissimilarities in preclinical development programs, as well as risk-based approach influence for considered non-clinical evaluations. Results showed that although ATMPs have specific characteristics, some non-clinical studies must be performed independently of the medicinal product’s features, on the other hand, certain should only be conducted if outcomes are considered relevant for ATMPs evaluation. Furthermore, it was possible to conclude and discuss common preclinical studies’ goals between different ATMPs, as well as relevance of standard preclinical evaluations when considering these innovative medicinal products. Moreover, importance and benefits of risk identification and appraisal, during non-clinical evaluations, were also discussed for selected case studies.

KEYWORDS: Advanced Therapy Medicinal Products, Cell-Based Therapy, Gene Therapy, Preclinical, First-in-Human, Non-clinical, Risk-Based Approach, Marketing Authorization Application; European Public Assessment Reports;

Resumo

Têm sido realizados esforços notáveis no desenvolvimento de novos medicamentos que permitem a alteração de modelos de tratamento standard para uma abordagem personalizada para cada doente. Os medicamentos de terapias avançadas, baseados em células, tecidos ou genes, oferecem soluções inovadoras para necessidades médicas não colmatadas, apresentando um grande potencial. Porém, devido à complexidade e singularidade das terapias avançadas, a regulamentação deve ser adaptada caso a caso de modo a avaliar eficientemente esta classe terapêutica e simplificando a sua entrada no mercado. Esta dissertação pretende discutir requisitos pré-clínicos, obrigatórios para a realização de estudos em humanos, focando-se em produtos baseados em células e recorrendo ao software NVivo para uma análise eficaz. Inicialmente, foram comparadas diretrizes relacionadas com os estudos não clínicos de pequenas moléculas, produtos biotecnológicos, terapias celulares e medicamentos de terapia génica. Em segundo lugar, foram analisados seis casos de estudo diferentes, avaliando semelhanças e diferenças em programas de desenvolvimento pré-clínico, bem como a influência de uma abordagem baseada no risco para a realização dos estudos considerados. Os resultados mostraram que alguns estudos não clínicos devem ser realizados, independentemente das características do medicamento e outros só devem ser executados se os resultados expectáveis forem considerados relevantes. Foi ainda possível discutir a existência de objetivos idênticos para os estudos analisados, bem como a relevância das avaliações pré-clínicas padrão na avaliação de medicamentos inovadores. Finalmente, a importância e os benefícios da identificação e avaliação de riscos, durante avaliações não clínicas, também foram analisados e discutidos considerando os casos de estudo selecionados.

PALAVRAS-CHAVE: Medicamentos de terapia avançada; Terapia Celular; Terapia Genica; Estudos pré-clínicos; Ensaios Clínicos; Autorização de Introdução no Mercado

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List of Abbreviations

ATMP	Advanced therapy Medicinal Product
EPAR	European Public Assessment Report
EU	European Union
FIH	First-in-Human
EMA	European Medicines Agency
FDA	Food and Drugs Administration
MAA	Market Authorization Application
SAD	Single-ascending Dose
MAD	Multiple-ascending Dose
CAT	Committee for Advanced Therapies
CHMP	Committee for Medicinal Products for Human Use
PD	Pharmacodynamics
ADME	Absorption, Distribution, Migration and Excretion
CBMP	Cell-based Medicinal Product
sCTMP	Somatic Cell Therapy Medicinal Product
W	Weeks
D	Days
N.S	Not Specified
MF	Microfracture
H	Hours
GCV	Ganciclovir
GvHD	Graft versus Host Disease
3D	Three-dimensional

1. Introduction

The insertion of a new pharmaceutical in the market is frequently an arduous procedure which requires tight regulation to assure safety and efficacy to the targeted population, independently of drug's pharmacological activity. The whole development process entails different steps, namely research and development, the preclinical, clinical and post-authorization phases. These stages must be tailored according to the pharmaceutical being developed and must comply with different regulatory documents [1].

Over the past decades, a great investment and progress has been seen when it comes to pharmaceuticals aiming to deliver a more personalized and innovative care, including cells, genes and tissues in their constitution, the so-called **advanced therapy medicinal products (ATMPs)**. However, such novel treatments are, in consequence of their patient-specific target and structure, more complex and heterogeneous. The development of these forefront innovative pharmaceuticals led to the necessity of specific guidance and legislation, tailored for this new biotechnology healthcare area [2].

As the basis of ATMPs are cells, tissues or genes, standard regulatory context may not apply during most of product development. Thus, it is important to follow specific regulatory documents and identify, according to advanced therapy's characteristics, the mandatory steps to be performed in order to identify safety, tolerability and feasibility of these innovative products' administration [3]. The majority of these pharmaceuticals' features are characterized during early stages of product development, particularly preclinical stage and first-in-human studies, to ultimately assure a high level of health safety and increasing benefit-risk ratio, before administrations in larger target groups [4].

Due to the need for unique regulatory documents, considering ATMPs characteristics and complexity, since 2008 regulation have been effectively adopted for these pharmaceuticals, namely *Regulation (EC) No 1394/2007* [5], aiming to help in product market insertion and in complex development program supporting market introduction [3]. However, there is still no scientific guideline particularly amending requirements for first-in-human studies, considering all ATMPs categories. The complexity of this healthcare area is clear when considering the diverse nature of ATMPs, for example when it comes to raw materials or manufacturing process and have led to the elaboration of different regulatory documents, from scientific guidelines to reflection papers, that must be considered when developing an ATMP [6]. Besides, many of these products are created in academia or small companies, which usually lead to the necessity of closer support when considering complex regulatory requirements for these pharmaceuticals.
Therefore, one can anticipate that ATMPs development face several concerns, hazards and limitations. The difficulties that this field poses are clear if one look at the number of approved ATMPs in EU, which still remain quite low [2].

Although this is an intricate therapeutic field, improvements have been made in trying to increase the number of available advanced therapy medicinal products, due to great value that these products

pose, since the majority aim to mitigate unmet medical needs and find solutions for life-threatening diseases and therefore posing potential treatment opportunities for diseases that, nowadays, don't have any successful therapeutic solutions. Thus, this major potential impact that ATMPs show to the healthcare community served as the core motivation for this study. After some literature review within the topic, it was clear that appropriate preclinical development steps were of key importance for these pharmaceuticals' market entrance, allowing to minimize clinical trials' risks and achieve a safer product evaluation process when starting clinical studies with first administrations in humans. These two aspects combined together led to the conduction of a comparative analysis with regards to preclinical steps, mandatory for first-in-human studies of ATMPs, with a particular focus on cell-based medicinal products, including somatic cell therapies and tissue engineered products, with the key goal of answering the questions "What are the relevant preclinical studies for ATMPs?" and "What are the similarities and differences encountered between different ATMPs preclinical steps?".

To perform a significant analysis, this study was divided in two major phases, allowing to acquire relevant information to conclude on different topics of the present thesis. Firstly, different scientific guidelines, with regards to preclinical development steps, were compared, relating product characteristics with relevant preclinical studies. Secondly, different ATMPs' European Public Assessment Reports (EPAR), particularly somatic cell therapies and tissue engineered products' EPARs, were analysed, preclinical studies were compared and discussed according to particular product features, such as mode of action or route of administration. Furthermore, outcomes obtained during previous study phase, relating different scientific guidelines, were considered throughout second stage discussion. Besides these two major phases, several research steps were conducted, each one addressing a different research question to be answered and serving as basis to acquire the desired outcome from this comparative study.

Moreover, this thesis was developed using a step-wise approach, allowing to use information obtained from the first steps as basis for the following ones. To simplify several documents' analysis and data organization the software NVivo was used. Furthermore, to facilitate readings and interpretation of this study, the present thesis is organized in different chapters, each one with a clear objective and a set of research questions to be answered.

The present chapter, *Introduction*, clarifies study design and the main drivers and goals set to conclude this thesis.

Chapter 2, *Methodology*, assess the way this comparative study was performed and the steps implemented to acquire the essential information required. The implementation of the aforementioned step-wise approach and the way NVivo software was used are explored in larger detail throughout this chapter. Also, the regulatory documents used as a basis for this study are named.

In chapter 3, *ATMPs and Drug Development Process*, an overview of Advanced Therapy Medicinal Products' context is provided, to answer the question *"What is an ATMP?".* Also, the current legal framework for drug's market entrance is examined, as well as a picture of development steps required for a marketing approval and a review of regulatory entities involved in the process, within the

European context. Furthermore, the risk-based approach is also analysed, in particular when it comes to its implementation during ATMPs development, as well as the advantages and requirements that its application poses. This methodology allows to develop an evaluation program for each ATMP with a product-specific methodology and consequently acquire more relevant outcomes based on the identified expected risks.

Across Chapter 4, *Preclinical Data Package supporting FIH*, the results obtained to answer the questions "*What are the mandatory information to be available before first-in-human studies?"* and *"What are the differences in preclinical data package required for standard drugs, biopharmaceuticals and ATMPs?"* are presented. To answer these interrogations a comparison was performed between scientific guidelines, assessing preclinical data package required for first-in-human studies, with regard to standard medicinal products, biotechnology-derived pharmaceuticals and ATMPs. This evaluation assessed similarities and disparities between considered guidelines. A further detailed appraisal was performed for each study identified in considered scientific guidelines.

In Chapter 5, *Case Studies Analysis*, the question *"What are the specific similarities and differences of preclinical studies among approved ATMPs?"* is answered and to do so different approved ATMPs' development programs are compared, as case studies for the evaluation of essential preclinical data package. The goal of this phase was to assess which information present in scientific guidelines and analysed in chapter 4 was considered for particular ATMPs' preclinical studies and if some similarities or differences can be found in development processes of different pharmaceuticals, considering that some of their features may be compared and others are distinct. In order to perform a meaningful analysis this study phase was divided in three sections. The first one intends to discuss the differences and likenesses between ATMPs with similar features, particularly the chondrocyte-based medicinal products. Furthermore, considering products' method of administration, expected mode of action and possible related risks, it was considered appropriate to divide ATMPs in different categories, with respect to their application mode and risks that they might pose, respectively a local or systemic administration and local or systemic risks. Results obtained for this ATMPs classification and further discussion within the topic are present in the second section of this study phase. Finally, the last section covers some general insights common between different non-clinical studies performed, even when considering very diverse ATMPs with distinct intended therapeutic outcome and mode of action.

Lastly, Chapter 6, *Conclusions,* encloses key conclusions of this study. Chapter 7, *References*, contains the references and sources consulted during the investigation.

2. Methodology

In this chapter the methodologies used to collect, organize and synthetize outcomes about the subject under study are presented. A brief description of the research tools used to identify useful information for further analysis and a justification on the use of the software Nvivo, as the approach used to help synthetize data, will be provided.

The methodology applied may be divided in different steps, in accordance with the expected outcome of each study's phase.

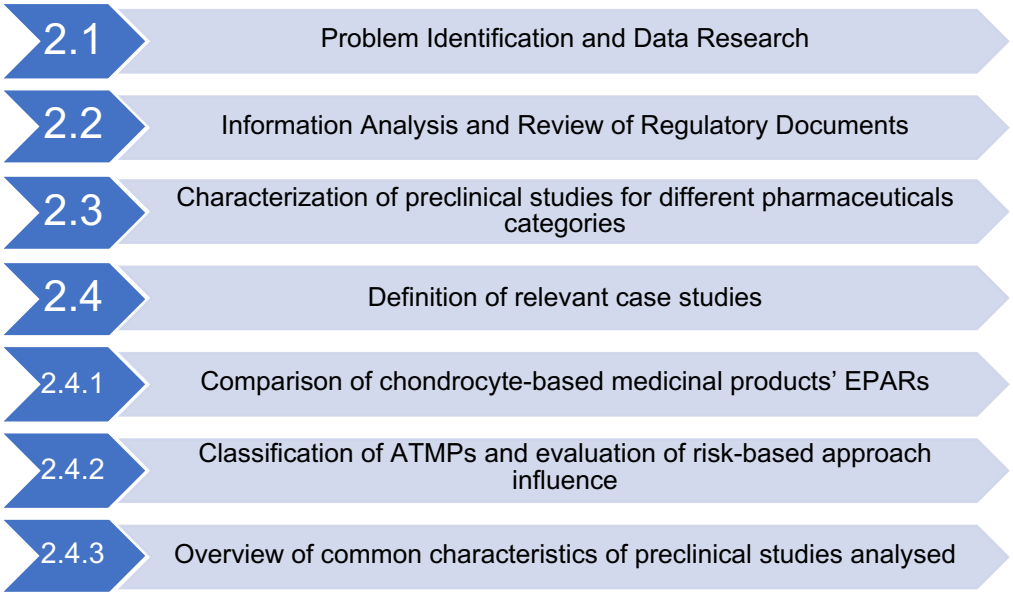


Figure 1 - Thesis development steps

2.1. Problem Identification and Data Research

The purpose of the literature research performed for this study was to acquire all available information related to the topic under analysis, with a particular focus on the European context and regulatory documents made available by European Medicines Agency (EMA).

Considering the complexity of new drug's market entrance and all the regulatory aspects mandatory to allow drug's approval, it was considered appropriate to use several research questions, some already named in previous chapter, to define all information that must be obtained. These questions were established as guidelines to select relevant documents for further analysis, thus only documents with information relevant to answer the proposed questions were selected.

The information source consulted was European Medicines Agency (EMA) website, and for some concepts also Food and Drug Administration (FDA)'s documents were considered, in order to have clearer understanding of less explored topics within European documents.

Thus, the principal research questions to be answered, throughout this thesis, are:

1) What is an ATMP?
2) What steps and information are mandatory for a drug's marketing approval?
3) What are the regulatory entities responsible for ATMPs evaluation within the EU context?
4) What is the mandatory information to be available before first-in-human studies?
5) Are there any differences in preclinical data package for different pharmaceuticals?
6) What are the differences and similarities of standard molecules, biopharmaceuticals and ATMPs' preclinical studies?
7) What are the already evaluated ATMPs so far?
8) What are the differences between diverse ATMPs development programs for non-clinical studies?
9) How risk-based approach influenced each ATMP non-clinical evaluation?
10) What risks can be identified for each ATMP and how they were assessed during non-clinical studies?
11) What is in common between all ATMPs non-clinical evaluations and how can it be compared with recommendations for preclinical analyses before initiation of first-in-human studies?

Bearing in mind the proposed questions, some keywords and expressions were used to identify relevant regulatory documents available in EMA website, particularly: *Advanced therapy Medicinal Products, Cell-Based Therapy, Gene Therapy, Preclinical, First-in-Human, Non-clinical, Risk-Based Approach, Marketing Authorization Application.*

Besides research in EMA website [7] by key word, also the sections Human Regulatory > Research and Development > Scientific Guidelines > Non-Clinical > Non-Clinical Development and Human Regulatory > Advanced Therapies > Scientific Guidelines were consulted, to assure that all relevant scientific guidelines, commission directives and reflection papers, as well as some presentations, were considered for further analysis.

2.2. Information Analysis and Review of Regulatory Documents

Since a large number of documents had to be considered, assessment of all information available became a demanding task. To facilitate readings and information synthesis software NVivo, from QSR International, was used. NVivo is a qualitative data analysis software that allows data storage, categorization, organization and management, offering information research tools such as queries and comparison diagrams [8].

Thus, the regulatory documents considered relevant for this thesis were uploaded to NVivo and the software was used as a tool to allow a more efficient and successful data analysis. The software was used throughout different steps of the methodological process, with a bigger impact during step 2.3,

for characterization of preclinical studies for different types of pharmaceuticals, and step 2.4 for comparison of different case studies.

When it comes to research outcomes explored in chapter 3, literature reviewing was useful to acquire broad-spectrum knowledge with regard to ATMPs related concepts, drug development process and regulatory context for drug's marketing approval within the EU.

2.3. Characterization of preclinical studies for different pharmaceuticals categories

After the primary literature review to acquire general knowledge within the topic, a more detailed analysis was performed with regard to different preclinical scientific guidelines. This phase aimed to answer the questions, already present in chapter 1, *"What are the mandatory information to be available before first-in-human studies?"* and *"What are the differences in preclinical data package required for standard drugs, biopharmaceuticals and ATMPs?".*

In order to define the preclinical study design, the product's characteristics, therapeutic goal and mode of action must be considered. Bearing in mind each product specifies, different recommendations and regulatory documents apply accordingly to the medicinal product being developed.

One can anticipate relevant differences between different types of pharmaceuticals, considering for example their origin or manufacturing processes, which leads to significant dissimilarities during the whole development process and consequently the preclinical stage [9]. This analysis allowed to assess why some studies are relevant for a particular type of drug and others do not apply. One particular example of how pharmaceuticals can pose so relevant differences in manufacturing process, mode of action or composition, which influence preclinical design, is the comparison between small molecules and biopharmaceuticals. Small molecules are chemically synthetized and show a well-defined structure. On the other hand, biopharmaceuticals are derived from living sources and show a more complex manufacturing process. Thus, taking into consideration the scope of this thesis it was considered relevant to analyze, compare and discuss different recommendations for preclinical studies of diverse medicinal products categories, keeping in mind the pharmaceuticals' characteristics.

Bearing these product-specific characteristics in mind, four scientific guidelines were studied and compared, assessing differences and similarities between them. For standard drugs, particularly small molecules, *ICH M3(R2) – Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals* [10] was considered, which gives a broad guidance to preclinical drug development process, particularly before initial drug's administration in humans. When it comes to biotechnology-derived pharmaceuticals, *ICH S6(R1) - Preclinical safety evaluation of biotechnology-derived pharmaceuticals* [11] was studied. With regard to advanced therapy medicinal products two documents were analysed, namely *Guideline on Human Cell-based Medicinal Products* [12] and the *Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products* [13], both

containing relevant information for the pharmaceutical' developers with regard to preclinical data package for marketing approval.

The essential data package for standard medicinal products, available in *ICH M3(R2)*, was included in a mind map created with NVivo software and represented in Figure 2. This map was also used for further analysis of case studies' EPARs.

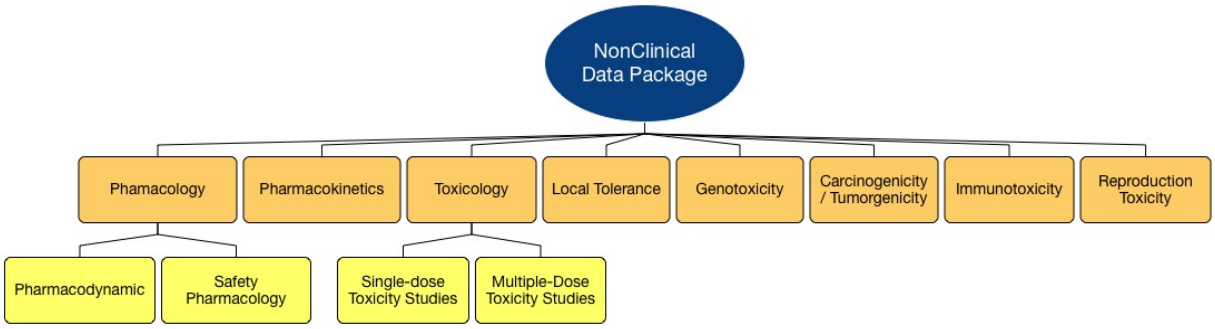


Figure 2 - Mind map with essential data package for standard medicinal products, during non-clinical studies (obtained using NVivo software)

Taking advantage of NVivo tools, nodes were automatically generated from the map, with the intention of understanding if the tests required for standard drugs are also referred in scientific guidelines amending the other three considered pharmaceuticals' categories, namely biotechnology-derived pharmaceuticals, cell-based ones and gene therapy medicinal products. Thus, a parent node was obtained for first order studies, represented in orange color. Child nodes were obtained for the tests represented in yellow and represent a subtype of study to be performed with respect to the parent non-clinical study type. With the nodes created, the four scientific guidelines for preclinical studies were coded and the presence of any reference to each one of these tests in each document was assessed, through a query performed in NVivo.

This methodology allowed a more efficient comparison between the four guidelines, as well as a clearer perspective of what are the similarities and disparities in preclinical requirements for different pharmaceuticals, considering each product's characteristics. The outcomes of this research step are addressed and discussed in chapter 4.

2.4. Case studies evaluation

The results obtained from the previous methodological step and present in chapter 4 allow to have a broad view of preclinical studies for different pharmaceuticals, correlating the pharmaceuticals' general features and the preclinical requirements for first-in-human administrations. Additionally, it was considered relevant to compare different assessment reports in order to have more specific examples of preclinical study designs and how the pharmaceuticals' characteristics influence the correspondent non-clinical data package.

In the most desired scenario the relevant case studies chosen would still be available on the market. Besides, it was required that these products showed some common features and also some dissimilarities to allow a meaningful comparison between preclinical studies performed. Accomplish these two requirements proved to be not feasible due to the very low number of ATMPs available. Thus, it was necessary to consider some medicinal products that have already been withdrawn form market. However, it is relevant to stand out that none of the relevant cases chosen were withdrawn due to safety reasons, making them still relevant to be used as case studies.

In order to study examples which already received the European marketing authorization, the EMA website was explored to acquire the assessment report (EPAR) necessary to conduct the comparison analysis. Within the website it is not possible to choose the pharmaceutical category that is relevant, and so it is not easy to find all ATMPs already evaluated by EMA, in order to determine the meaningful ones. To help in this research, the document *Advanced therapy medicinal products (ATMPs) and ATMP Regulation* for the 2nd International Awareness Session – The EU medicines regulatory system and the European Medicines Agency, by Patrick Cells (8 March 2018) [14] was used to know all the marketing authorizations obtained for ATMPs until December 2017. Therefore, in the EMA website, particularly in the section Find Medicine > Human Medicines [15] the medicinal products were searched.

Furthermore, to determine the relevant case studies for further analysis, the pharmacotherapeutic group, the ATMPs sub-category and the therapeutic indication were compared to assess which products showed similar features and which presented some distinct characteristics, to allow a significant comparison. ATMPs considered adequate for further analysis are assessed in chapter 5, where a brief overview of each one is available.

After identification of the relevant ATMPs to conduct a significant comparison of preclinical studies, the information present in each medicinal product's EPAR was summarised and analysed, using NVivo as a research tool to help identify the key points to gather meaningful conclusions and perform a more efficient analysis.

The outcomes of the present methodological step are depicted in Chapter 5.

2.4.1. Comparison of chondrocyte-based medicinal products' EPARs

From the evaluation conducted for each relevant ATMP's assessment report, it was considered relevant to create a category including three ATMPs studied. Thus, ChrondroCelect, MACI and Spherox were classified as chondrocyte-based medicinal products, due to their similar expected therapeutic outcome, mode of action and method of administration, further discussed in section 5.1.

With NVivo software, using a similar methodological process to the one discussed in *2.3. Characterization of preclinical studies for different pharmaceuticals categories,* the case studies

comparison was performed. The EPARs, of the three selected case studies, were imported to the software and coded using the same nodes as the ones used for previous analysis and obtained from the mind map present in Figure 2. Moreover, this three ATMPs were compared in detail for the different preclinical study categories identified, and also outcomes of scientific guidelines assessment, explored in chapter 4, were also considered. Some non-clinical studies' features discussed are related to studies' duration and animal models used for each preclinical data package considered.

The outcomes of these comparison process are present in Chapter 5, section 5.1.

2.4.2. Classification of ATMPs and evaluation of risk-based approach influence

Besides the comparison conducted for chondrocyte-based medicinal products, all six ATMPs considered as relevant were classified according to their method of administration and possible risks inherent to their used. Further from this classification, also the risk-based approach was kept in mind during the discussion conducted for the categorization attributed to each ATMP, evaluating the influence of this methodology implementation in each ATMP's development process.

The outcomes of these comparison process are present in Chapter 5, section 5.2.

2.4.3. Overview of common characteristics of preclinical studies analysed

After assessment of similarities and differences for chondrocyte-based medicinal products and classification of six analysed ATMPs, it was considered relevant to perform an overview of major common preclinical features identified, independently of the ATMPs' characteristics. Besides the insights acquired from the comparative study performed for the case studies, also the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16] was considered. Although ATMPs are not in the scope of this document, common points were identified when it comes to relevant studies required and so it was considered relevant to compare them with the outcomes of the case studies analyses performed.

3. ATMPs and Drug Development Process

3.1. Advanced Therapy Medicinal Products

Advanced Therapy Medicinal Products (ATMPs) is a ground-breaking pharmaceutical field aiming to restore, correct or modify physiological functions, through a metabolic, pharmacological or immunological pathway [17]. This emerging biomedical area comprises different subtypes, all of them either based on tissues, cells or genes [18]. Somatic cell therapies use cells and tissues which have a different function, biological features or structural properties in a recipient, according to their intended clinical use, in comparison to its essential function in the donor, who can be the same person. With the manipulation that these cells or tissues undergo it is possible to cure, prevent or diagnose diseases, through an immunological, pharmacological or metabolic process [19]. Other ATMP subtype is the gene therapy medicinal product, in which the use of recombinant genes inside the body leads to a therapeutic, prophylactic or diagnostic effect. Diverse products are included in this section, such as plasmid DNA, viral and non-viral vectors, genetically modified viruses and cells [20]. Finally, a tissue-engineered product entails cells or tissues that have been engineered in a way that allow them to repair, replace or regenerate human tissue, without identical essential purpose in the recipient and in the donor [21]. The active substance of these products may have different levels of differentiation, from an immature cell preparation to a differentiated tissue. Additionally, cells or tissues may be combined with one or more medical devices, characterized for that reason as a combined ATMP [22].
Due to these therapies' biotechnologic background, each new ATMP developed is very specific and distinct from other products that might be already available [23]. For that reason, it is important to assess all product characteristic's, defining possible hazards for human safety, as well as identify differences in product nature that will lead to a change in significant tests performed throughout preclinical and clinical stages. Thus, quality, preclinical and clinical data required for pharmaceutical's evaluation is highly product-specific, which also requires a very expertise personnel responsible for the evaluation of product development, determining a positive or negative opinion towards it [5].
Considering ATMPs specificity, a development system that assures complete traceability is crucial, allowing to trace, from the donor to recipient and vice-versa, all product components, such as starting materials and raw materials that may be in contact with the product, through sourcing, packaging, manufacturing and storage [24]. Besides, it is also crucial to consider non-product factors, which may influence the anticipated outcome, such as material compatibility and surgical procedure [21].

All information that must be available for drug's marketing approval, intends to describe and justify the whole product development process, product specific characteristics, as well as all tests performed for its complete evaluation with regards to safety and efficacy. This broad data package required for a marketing approval includes information related to product pharmacological class, intended mode of

action, proposed clinical use, manufacturing process, product characterization and quality, non-clinical and clinical reports, among others [19].

An example of ATMPs specificity can be provided with somatic cell therapies, which may give respect to stem-cell derived products as well as mature differentiated cell-derived therapies. Besides, cells may have an autologous, allogeneic or xenogeneic source and when it comes to stem cells their sources may be adult, perinatal, fetal or embryonic tissues. All of this variability of starting materials for cell therapies allows to illustrate ATMPs complexity when it comes to development and further evaluation [9].

Considering cell therapy, which are the foremost considered ATMP type in this study, there is a need for its characterization regarding structural, functional and biological features. To be possible to do so, cell origin, proliferation capacity and manipulation level, among others, must be assessed in order to define the more suitable evaluation design, evaluating safety and efficacy for human administration. In these therapies, cells can be used with other materials that must also be analysed to determine its safety. Furthermore, also materials used throughout development process for cell collection, culture or alteration must be evaluated. The therapy must be characterized according to cell origin (autologous, allogeneic or xenogeneic), level of manipulation, proliferative capacity, interaction with other cells or tissues and its proposed use. During quality assessment, therapy identity characterization gives information on phenotypic or genotypic profile, purity determines the quantity of viable cells and non-viable cells and potency data assesses product biologic activity in comparison to the intended use. Also cellular components' functionality and viability must be considered, as well as potential impurities [12].

When it comes to gene therapy, it involves an expression system in which a vector is used to genetically modify a cell. For these therapies it is important to characterize product expression in target cells and also assess these cells characteristics. Besides, source, manipulation and verification of the encoding gene sequence must be determined, as well as the vector being used, according to physico-chemical features [19].

Particularly for the purpose of this study, specific preclinical studies and tests required to determine ATMPs safety and efficacy for human administration, as well as product's characteristics that influence this data package will be analysed in further detail in chapter 4 and specific case studies will be analysed throughout chapter 5.

3.2. New Drug in the Market

The entrance of a new drug in the market is a complex process which requires money and time to accomplish the better outcome to the patients and industry.

The process steps needed for a drug entrance in the market may be divided in 4 major phases [25]:

1) Research & Development;
2) Non-clinical Development, which usually takes from 3 to 6 years;
3) Clinical Development, ranging from 6 to 7 years and embracing phase I, II and III of clinical trials;
4) Post-Approval Life-cycle management & Pharmacovigilance, during 2 to 5 years, including phase IV clinical trials.

Figure 3 - Medicines Market Entrance Process[26]

3.2.1. Research and Development

During this first stage of a new drug development, numerous compounds are candidates to become a new drug in the treatment of a specific condition, with the goal of identifying the most promising candidates for further development. Diverse tests and evaluations are performed, using *in vitro* testing models, to identify the best candidates and furthermore, to study compound's mechanism of action, pharmacokinetics, potential benefits, mode of administration and effectiveness when compared to other drugs for the same condition, already available in the market.

From the numerous candidates for a new drug, only one goes further in evaluation process, going through non-clinical evaluations and reaching human studies. In order to success in preclinical studies and start clinical evaluations, there has to be information regarding anticipated risks and reactions based on the pharmacological and toxicological data obtained from *in vitro* and *in vivo* studies performed in animal models [27].

3.2.2. Non-Clinical Development

The non-clinical development phase aims to study the safety and efficacy of the new candidate drug being tested, characterizing potential adverse effects that might occur under conditions in which the drug is intended to be used, as well as validate proof-of-principle. The data acquired throughout this stage is imperative to assure positive outcomes from drug's use and decrease risks to which the individuals are exposed during clinical phase and after drug's market approval [10].

Throughout this stage, *in vitro* and *in vivo* tests can be performed to have a better insight of drug's potential, with the results of non-clinical investigations indicating if the drug is acceptably safe to conduct further investigation in humans.

Some factors influence the nature of studies performed during this development period such as the characteristics of the drug under development, the targeted condition, the target population and the route of administration that is intended to be used [10].

The non-clinical safety and efficacy assessment include pharmacodynamics, pharmacokinetic and different toxicity studies. These analyses provide information related to drug's mechanism of action, dose-response relationship, potential route of administration and general pharmacology [10].

One of the most important parameters to study is the dose administered, which choice must be justified taking into consideration non-clinical studies performed, assessing the selection of the initial dose to administer in humans, the safe dose exposure time and the most adequate mode of administration [10]. Furthermore, studies must be performed under Good Laboratory Practice regulations, allowing researchers to review their findings and decide whether the drug should be tested in humans [28].

In case serious adverse findings are obtained, during non-clinical development, the study design might be altered and ultimately, studies might be abandoned in case the decision is justified by the new data acquired. When developing a new pharmaceutical for a life-threatening or serious disease the non-clinical approach should be developed in a case-by-case basis [10]. If the drug under appraisal is biotechnology-derived or an advanced therapy medicinal product, standard battery of non-clinical studies might not be the most suitable, not allowing to obtain meaningful information about the new product which turns them insignificant when deciding whether to carry on with medicinal product analysis. Therefore, a more individualized approach is required, with the use of a specific set of studies, taking into account the origin and characteristics of the new pharmaceutical [11].

Currently, it is increasingly critical to obtain a harmonized approach for the implementation of preclinical studies during the development of a new pharmaceutical, particularly among different world regions. For that reason, to facilitate the adoption of a scientifically and ethically appropriate study design and promote safety and efficacy of a new pharmaceutical, the *ICH M3(R2) - Guideline on non-clinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals* was developed in order to define the current recommendations regarding non-clinical analyses for standard drugs, particularly small molecules [10].

The conduction of proper non-clinical studies is of major importance, allowing to define pharmacological and toxicological outcomes predictive of human reaction, to reduce risks to which humans might be exposed during clinical development stages, to better comprehend the new pharmaceutical under evaluation and to have clearer insight of the risk-benefit that the intended approach represents to the target population. Hence, *ICH M3(R2)*, as well as recommendations for biotechnology-derived pharmaceuticals (*ICH S6(R1)*) and for advanced therapy medicinal products, for which a single harmonization document is still not available, will be analysed in larger detail throughout Chapter 4.

3.2.3. Clinical Development

Non-clinical studies give a first overview about pharmaceutical's mode of action and characteristics. However, to assure safety and effectiveness to the target population, a new drug must also undergo a clinical development stage in which the interaction with the human body is tested [29].

The clinical trial is developed with the purpose of obtaining the most relevant information possible to assure the best benefit-risk ratio to the target population and improve patients' quality of life after the use of the medicinal product under evaluation. During this stage of drug development, non-clinical studies may still be conducted. In case non-clinical and clinically relevant data emerges, regarding drug safety, the information about the pharmaceutical must be reviewed, as well as the study design adopted, taking into consideration the new evidence obtained [30].

The clinical process can be described as a "step-wise procedure", in which information acquired from previous studies is considered to support and establish the next steps in drug evaluation [30]. The most adequate development plan for the new pharmaceutical can be traced considering drug's profile which is possible to build based upon the information already available. First studies performed aimed to better comprehend short-term safety and tolerability of the new drug. On the other hand, trials that are implemented later include a more diverse population, have a confirmatory intention and include a larger population percentage [1].

The design of a clinical trial is an important step, which will allow to obtain the required answers to some questions and issues about the new drug. Based on the information that is already available before the initiation of the clinical phase, the researcher must decide some features of the protocol, such as the individual's profile that is most suitable to be endorsed in the clinical trial, how many people will take part, how long will it be or the data that will be evaluated or reviewed. After the definition of these important process's features, trials can be initiated [29].

Phase I Clinical Trials

Phase I clinical trials aim to study human pharmacology and assure safety as well as define dosage. This stage usually includes 20 to 100 healthy volunteers and takes up to 7 months. During this period the volunteers are closely monitored and researchers collect information on drug's process inside human body [30].

Studies conducted during phase I clinical trials have no therapeutic purposes and involve the estimation of initial safety and tolerability as well as the nature of adverse reactions that might occur. These data can be obtained through single ascending dose (SAD) and multiple ascending dose (MAD) studies. In single ascending dose studies if subjects do not show any relevant negative outcomes and results are aligned with expectations, the dose can be escalated, and a new group of individuals receives a higher dose. Regarding multiple ascending dose studies, pharmacokinetic and

pharmacodynamics data is obtained considering the multiple dose design. As occurs in SAD studies the dose in incremented in the following groups [1].

Moreover, information regarding pharmacokinetics is also obtained. Within this class, absorption, distribution, metabolism and excretion data is included and by gathering this knowledge, the clearance of the pharmaceutical can be studied, which is considered relevant to estimate the dose to be given to the individual as well as dosage's frequency. Besides pharmacokinetics, data related to pharmacodynamics is also acquired and allow to earlier estimate drug potential efficacy and activity inside the human body. These studies might not be appropriate when considering advanced therapy medicinal products and tests performed must be tailored in order to fully study drug's potential and properties [12].

Nevertheless, there is an increasingly recognized trend in the conduction of an Exploratory Clinical Trial, intended to be performed in early phase I clinical trials. This approach allows an initial contact with human data, using limited human exposure and no therapeutic intent. Furthermore, this methodology does not need the same non-clinical data package that is demanded for a traditional phase I clinical trial and allows the reduction of risk exposure in FIH studies [10] .This approach is a significant change in clinical development of new pharmaceuticals that has been verified in the past few years, and it is very well accepted among the scientific community due to ethical concerns around human drug's testing as well as high development costs needed during clinical testing phases [31].

Taking into consideration the goal of this thesis, regulatory context behind preclinical data required for the initiation of studies in humans will be further analysed throughout chapter 4 and 5.

Phase II Clinical Trials

When it comes to phase II clinical trials, the purpose of this development period is to assess drug's efficacy as well as understand side effects that the pharmaceutical may cause to the human body. During this stage, the drug is tested in several hundreds of individuals that suffer from the disease targeted by the new pharmaceutical and it may take from several months to around 2 years [29]. In this clinical development point, the patients included in the trial are chosen with respect to constricted criteria, allowing to have a homogenous testing population. Through the use of dose escalation designs, dose and regimen to be used in phase III trials can be tested, as well as some potential study endpoints, which allow to obtain critical information during drugs testing [30].

Phase III Clinical Trials

Concerning phase III studies, the core purpose is to confirm safety and effectiveness of the new pharmaceutical in the target population, thus providing therapeutic benefit to patients. During this stage a confirmation of previous data gathered throughout testing procedures is accomplished and serve as a basis for marketing approval. It is during phase III that a wider population is tested, as well

as different disease stages, allowing to have a broader description of pharmaceutical's characteristics [30]. During this stage thousands of individuals that suffer from the targeted condition are included and monitored for a longer period, up to some years, during which it is possible to detect adverse reactions that have remained unknown so far [29].

3.2.4. Post-Approval Life Cycle management & Pharmacovigilance

After drug's approval by regulatory authorities, phase IV clinical trials are initiated. This is possible since tests performed during this stage are not considered mandatory for drug's marketing authorization but are critical for optimizing treatment outcomes and possibly refine dosing recommendations, aside from defining a general benefit-risk assessment and identify long-term side effects [32].

The pharmacovigilance and risk management plan are implemented in this pharmaceutical's life period, in order to provide information on drug's outcome when the pharmaceutical is being used under normal practice conditions, with a less controlled environment, allowing to trace an adverse event profile. Throughout this stage, the risks of adverse reactions are detected, assessed, minimised and communicated [33].

3.3. Regulatory Framework

During the last few decades the regulatory framework for drugs entering the market has seen an enormous transformation, with the harmonization of the regulatory approval process being increasingly required [6]. Guidelines from European Medicines Agency (EMA) and Food and Drug Administration (FDA) were created and modified in order to accomplish the synchronization in drug development process, bridging gaps between studies performed throughout non-clinical and clinical phases, in different world regions [34].

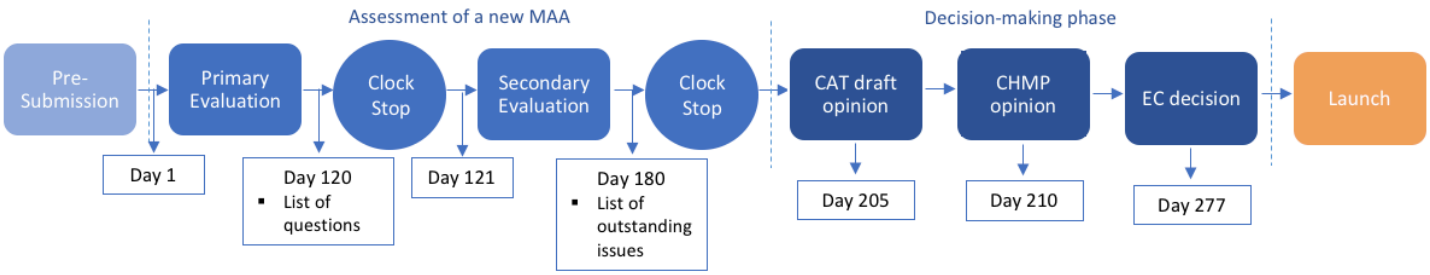
With a focus on ATMPs, the legislation developed in a way that these therapies' market entrance was made easier, to help increasing the number of these medicinal products available for patients. A centralised procedure is mandatory when acquiring a marketing authorisation for ATMPs. With this approach, a single document is submitted, a Marketing Authorisation Application (MAA), that once granted it allows the accessibility to the therapy in different countries within the European Union, providing a free movement of authorized medicinal products [3].

The entity responsible for drug's market entrance approval is the European Medicines Agency (EMA), together with its scientific committees, particularly CAT, the Committee for Advanced Therapies, established in 2009, due to the increasing necessity of a specialised body for the evaluation of ATMPs' submissions. This board is responsible for the assessment and draft opinion regarding submitted MAAs, that are afterwards discussed within the Committee for Human Medicinal Products (CHMP) to

gather a conclusive opinion [35]. Since market authorisation is only granted if there is a relevant scientific assessment demonstrating quality, safety and efficacy of the medicinal product under evaluation, it is of crucial importance the scientific assessments performed to allow the demonstration that the benefits surpass the risks associated to the medicinal product's administration [36].

The scientific evaluation usually takes 210 'active' days and includes the revision by CAT and the final opinion given by CHMP. During the evaluation process, the active time is interrupted by at least one clock-stop period, during which the applicant prepares the answers to CAT questions. This stopping period might occur after day 120, when a list of questions is adopted and after day 180, next to the adoption of a list of outstanding issues. Before CAT embraces an opinion, an oral explanation usually takes place, during which the marketing authorization applicant address questions that have been raised about the product under evaluation [37].

The scientific evaluation stage can be divided in different steps as illustrated in the following scheme [14]:



When considering ATMPs, the regulatory framework differs from usual medicines available in the market. ATMPs' development considers the risk-based approach as a legislative background, to assure minimisation of risks to which population might be exposed. With this method, the developers are capable of define and support the extent of quality, non-clinical and clinical data that has to be included in the MAA submitted to appraisal, keeping in mind a risk profiling method, in which risks and risk factors are identified during product's development [38]. Bearing in mind the importance of the risk-based method in ATMPs' development and legislative context, this approach will be further analysed in greater detail in chapter 5, section 5.2, with discussion of its implementation in specific case studies processes.

The complexity of ATMPs therapeutic field leads to the necessity of precise legal definitions, due to product's specifications, in order to grant marketing approval [6]. Thus, to cover all ATMPs' characteristics and supress hazards to human's safety, some directives and regulations must be respected. *Directive 2001/83/EC* [39] regulates all medicinal products for human use. Particularly, part IV of Annex I is related to ATMPs field, as amended by *Directive 2009/120/EC* [19], documenting requirements for non-clinical, clinical and quality data. After 2007, *Regulation 1394/2007* [5] allowed to

place under the same regulatory context the three different ATMPs subtypes, named previously, as well as provide a certification system for this scientific field. Such certification allowed academic research groups and small companies, mainly responsible for ATMPs development, to guarantee conformity in preclinical and quality aspects of development with the regulatory context, which attracts capital to finance further development steps, namely clinical trials [36].

Bearing in mind that these products' development process must be in compliance with Good Manufacturing Practice, guidelines to accomplish so are provided in a document from November 2017 [40].

Additionally, regulatory documents related to cells and tissue donation, preservation and processing [41], as well as reports from expertise group meetings and risk-based approach recommendations are emerging to tightly regulate this area that represent a nascent field within medicinal products.

3.3.1 Marketing Approval Timeline – Alofisel

Studying a specific ATMP, Alofisel, a cell-based ATMP obtaining a positive opinion by the end of 2017, it is possible to illustrate the several stages throughout product approval. In July 2015 Alofisel was presented as a new application for evaluation, two months later, in September, it was nominated for peer review and rapporteurship. Later in 2015, the company asked for a delay in the MAA submission until 2016. In July 2016, it was submitted to questions and the rapporteurs presented an initial assessment of the MAA. In September of the same year the company asked to extend the clock-stop in order to respond to the list of questions. Later, in February 2017, a list of outstanding issues was given and in May the company asked again for the extension of clock-stop to answer the pending questions. Later, in October 2017, the rapporteurs presented the assessment of the responses from the applicant to the latest list of outstanding issues. Finally, in last December, satisfactory responses were provided regarding outstanding clinical issues and a positive CAT opinion was adopted by the majority with 28 members' votes in favour and 2 against. Besides, CAT agreed that all pending quality issues can be resolved in post-authorization phase. The CAT assessment report was adopted and transmitted to the CHMP [42]. The whole marketing approval process took almost two years and half.

3.4. Context

Advanced therapy medicinal products field have been developed during last decade, granting encouraging opinions, with some submissions being launched to the market. To have a clearer picture of this evolution, data was obtained from CAT monthly report of September 2018 [43]. In figure 5, it is possible to observe the percentage of ATMPs that were submitted, received positive opinion, were withdrawn or received a negative opinion, from 2009 until September 2018 within the European context. From the analysis of this data one can understand the positive trend in the higher percentage of ATMPs that received a positive opinion in contrast with the ones granting a negative one. Besides, from the number of withdrawals that are shown, some indicate products that had positive outcomes

but faced other concerns, such as economic or manufacturing site issues and for that reason were excluded from market.

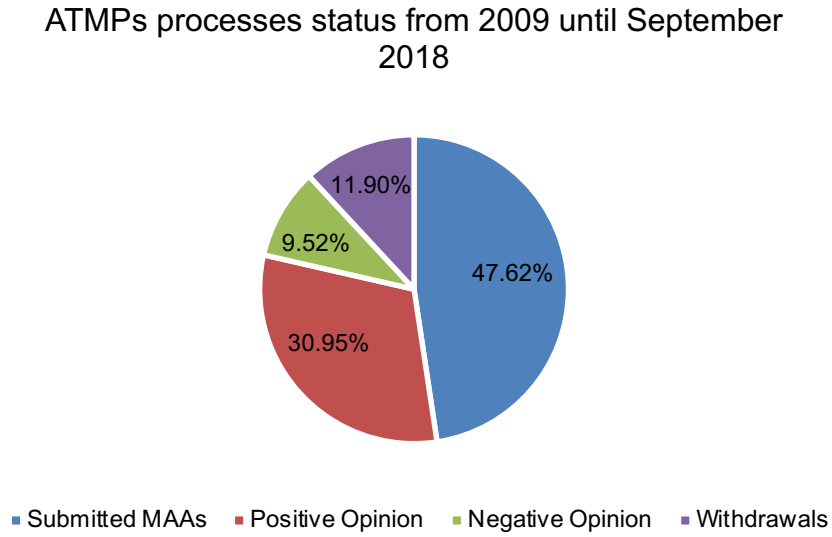
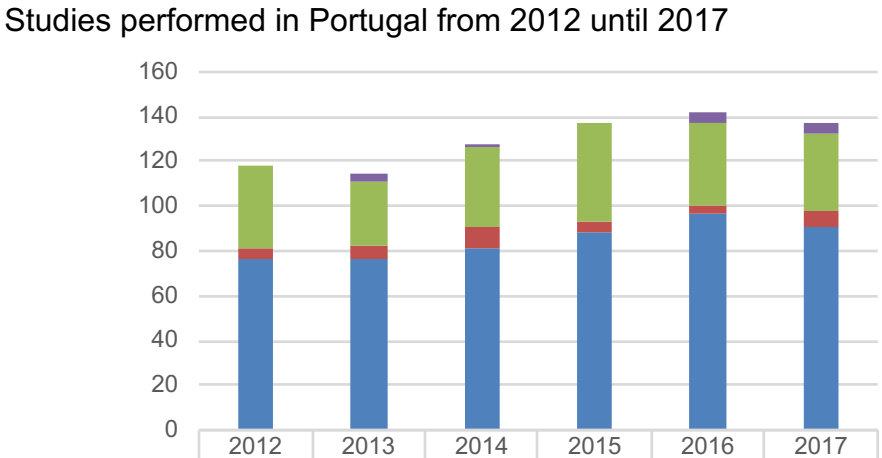


Figure 5 - Percentage of ATMPs according to its position on the market (submitted MAA, positive opinion, negative opinion, withdrawals)

When it comes to Portugal's framework the evaluation is conducted by comparing the studies performed for the different pharmaceutical categories. Fortunately, the same positive trend in ATMPs' evolution is verified, being illustrated in figure 6. The data present in this graph was obtained from Infarmed annual evolution report 2017, available online and altered using Microsoft Excel tools [44].



	2012	2013	2014	2015	2016	2017
Advanced Therapy IMP	0	3	1	0	5	4
Biological / biotechnological origin	37	28	35	44	37	35
Chemical & Biological / biotechnological origin	5	7	10	5	3	7
Chemical origin	76	76	81	88	97	91

From the information present in the figure 6, it is possible to understand the increase in ATMPs' studies within Portugal. Looking at the types of tests performed in the country, in 2012 and before, ATMPs showed no occurrence. On the opposite, when it comes to 2016 and 2017 there were already 5 and 4 ATMPs studies taking part, respectively.

Within ATMPs, different therapeutic categories show difference percentages when looking to the number of clinical trials being performed. In a study, from 2016, Hanna et al. identified almost one thousand clinical trials regarding advanced therapy medicinal products and among these studies, around 54% were somatic cell therapies, 22% were tissue-engineered products, 22% were gene therapies and 1,2% were related to combined products [45].

Besides, EMA have also shown a positive trend in ATMPs' development since CAT creation in 2009, with 287 scientific procedures and 291 scientific recommendations for ATMPs' classifications, according to March 2018 CAT monthly report [46]. These higher numbers contrast with the quite low quantity of ATMPs available on the market, which can be justified with the difficulty in these products' developments, due to their complexity, not standardised manufacturing procedures and patient-specific outcomes as well as economic issues, since these therapeutic solutions are very expensive, and the majority present a relatively small target population.

Until September 2018, only 12 ATMPs have entered the European Market. Provenge, Zalmoxis and Alofisel classified as cell therapies, Glybera, Strimvelis, Imlygic and Luxturna, the last one approved during September [47], classified as gene therapeutic products, as well as ChrondroCelect, MACI, Spherox and Holoclar, considered tissue-engineered therapies [14].

From the value that these therapies show to the population, with solutions for life-threatening and unmet medical conditions, we consider to be important to increase the number of on-going clinical trials as well as the number of approved products. Doing so can be a complicated task, considering pharmaceuticals' specific characteristics and mode of action, however an effort in regulatory harmonization is needed to support this complex product development.

3.5. Risk-Based Approach

Among the healthcare personnel and pharmaceutical industry there have been an increasing tendency in trying to efficiently acquire better outcomes when it comes to healthcare services or products. Concerning new drugs available on the market, this is not always easy to do so. Although the preclinical and clinical phases of development allow to obtain sufficient information on drug's mode of action or response, this data is not enough to clearly state all possible scenarios when the treatment is being used on a daily basis, within the healthcare services. Drug usage's drawbacks that might be verified might lead to a step back in drug's development and in an extreme case may even obligate to its' market removal. Thus, a new method is required to improve system's and product's quality in an efficient way, reducing development expenses and eliminating unexpected jeopardies. In an attempt to

identify and reduce risks which may provoke worse outcomes, since some years ago, a risk-based approach has been stated has the emerging methodology to assure systems quality, not only when considering medical care, but also in finances or banking [48].

The risk-based methodology is centered around the idea of inducing regulatory flexibility, by continuously monitoring risks and risk factors, with an early identification of hazards, that allows to control and minimize them in the risk management context. Moreover, with this method it is possible to specify the extent of non-clinical, clinical and quality data needed to be included in the MAA [38]. Hence, it can be defined as a proactive appraisal, using prevention as an essential basis and leading to an increased benefit-risk ratio to patients, with the assurance that benefits surpasses risks by the maximum possible margin [49].

The methodology is focused on risks defined, by EMA, as an adverse effect of concern to the patient and third parties and which is related to drug's clinical use. The quantitative and/or qualitative product's characteristics that contribute to a specific risk is defined as a risk factor [38]. From these two notions, the principle of risk profiling arises, with a characterization of each risk that is related to the product and not the overall product's risk. The risk profiling idea comprises four steps which allow to integrate all data available to design a profile to each hazard identified. The methodology is depicted in figure 7 allowing to clearly understand the model.

1st Step: Risk Identification	Throughout this phase any relevant risks are identified, which should start as early as possible in drug's development steps. The risks associated to each new medicinal product are related to product's nature, manufacturing process or mode of administration; During this step the question "What could go wrong?" must be answered.
2nd Step: Risk factors identification	During this second period the risk factors inherent to each risk must be identified, allowing to comprehend the causes of a specific hazard. These risk factors might be related to product's quality, biological activity and application and one specific risk factor may contribute to different risks and be interlinked between one another.
3rd Step: Map relevant data	Mapping the significant data for each risk factor associated with a specific risk, allows to obtain a well-defined causative relationship between risks and risk factors. This information must be mapped in a two dimensional table that could be scanned for establishing associations between hazards and their causes.
4th Step: Risk-Risk factor relation	Through this step the relevant risk-risk factor relationship must be well addressed in a narrative test which must include the causative science-based relation, a description of performed studies to acquire the connected relations and a conclusion on whether the scientific information available is enough and adequate to support MAA.

4. Preclinical Data Package supporting FIH

Prior to the conduction of clinical studies, where the product under development is already administered to humans, it is mandatory to acquire a data package that allows to assess if it is reasonably safe to do so. This preclinical information acquired, with the use of adequate *in vitro* and *in vivo* models, serve as basis for the next development steps, through a step-wise approach, allowing to conduct evidence-based decisions [6]. Thus, these preclinical studies must allow to obtain biological reactions similar to the ones expected for humans.

The objectives of non-clinical studies may not alter according to the medicinal product considered, being similar for small molecules or ATMPs. However, significant variations are clear when looking at the studies design to acquire meaningful outcomes, according to the medicinal product class [3]. Taking into consideration the specific characteristics that each pharmaceutical can show, particular requirements will apply, making this process product specific and different from pharmaceutical to pharmaceutical [38]. Although there are preclinical studies' features that must be considered in a case-by-case basis, similarities can be found between different development designs, starting with the purpose behind these studies. The ultimate goal of non-clinical procedures is to acquire the greatest amount of information possible, with regards to drug's features, proof-of-principle and possible adverse effects once administered in humans, allowing to obtain information for human risk prediction. Moreover, a primary safe dose and following dose escalation schemes are studied, as well as toxic effects in target organs, route of administration and relevant endpoints to obtain meaningful information throughout evaluation process [10]. Furthermore, these studies must also allow to obtain data related to duration of exposure and follow-up, which must allow to observe possible adverse effects [12]. Therefore, independently of which pharmaceutical is being analysed, these non-clinical studies must assess safety and efficacy of the new drug, while guaranteeing animal's minimal utilization. This is accomplished through the combination of *in vivo* and *in vitro* studies, which allows to conduct a more accurate extrapolation to human expected findings [11]. The use of *in vitro* studies is relevant for most pharmaceuticals, allowing to complement animal studies' outcomes and predicting useful effects in humans. For ATMPs' development programs, *in vitro* studies are even more significant, since relevant animal models may not exist, whereby these studies allow to acquire key data for further tests to be performed [50].

Bearing in mind this thesis goal, it is important to comprehend that these innovative products' characteristics will strongly influence preclinical studies to be performed. Advanced therapy medicinal products exhibit very particular characteristics, mode of action and target disease indications, which leads to the necessity of a particular preclinical data package to proceed to clinical development phases. Besides the specific nature of such drugs, ATMPs are most of the times result of innovative manufacturing processes or include components in their structure that have not been tested yet and

for which there is still no knowledge about risks and features. Thus, not only information and tests required to evaluate ATMPs differ from common pharmaceuticals approved, but also the data package has to be larger to characterize all product's features [9].

From the analysis performed with the software NVivo, aiming to compare scientific guidelines already named in section 2.3, *ICH M3(R2)* [10], the *ICH S6(R1)* [11], the *Guideline on human cell based medicinal products (EMEA/CHMP/410869/2006)* [12] and *Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)* [13], it was possible to reveal some patterns in preclinical data package which is required for first-in-human administrations, even though these scientific documents are intended to be considered for pharmaceuticals of diverse categories.

From that analysis, a matrix was obtained, illustrating the presence or absence of references to a particular study in each document. The number 1 ascertains the codification of the document with a particular node, representing that there is a reference to that type of studies in the document analysed. On the other hand, the number 0 determines that no part of the text was coded in the specific node and so there is no reference to that type of study in the document. The acquired matrix is represented in table 1.

Table 1 - Comparison table of different preclinical studies and scientific guidelines (obtained from NVivo)

	A: ICH M3(R2)	**B: ICH S6(R1)**	**C: CHMP/410869/2006**	**D: CAT/80183/2014**
1: Pharmacology	1	1	1	1
2: Pharmacodynamics	1	1	1	1
3: Safety Pharmacology	1	1	1	1
4: Pharmacokinetics	1	1	1	1
5: Toxicology	1	1	1	1
6: Single dose toxicity studies	1	1	1	1
7: Repeated dose toxicity studies	1	1	1	1
8: Local Tolerance	1	1	1	1
9: Genotoxicity	1	1	1	1
10: - Carcinogenicity/Tumorigenicity	1	1	1	1
11: Immunotoxicity	1	1	1	1
12: Reproduction Toxicity	1	1	1	1

Through this table analysis it is clear to comprehend that in all the considered scientific documents some reference can be found to each of the studies represented as a node which are obtained from ICH M3(R2), since all matrix entrances are “1”. This result obtained leads to the conclusion that independently of the sort of pharmaceutical under development it is necessary to consider the same non-clinical data package. Thus, independently of the medicinal product under evaluation it is required to discuss the relevance of all non-clinical studies categories considered essential for standard drugs, deliberating whether each study category is relevant to be conducted.

After acquiring this outcome, using Nvivo and keeping in mind pharmaceuticals’ specificities it was considered relevant to perform a more detailed analysis of the information coded at each node for the four documents, analyzing particular requisites for each pharmaceutical type. Therefore, additional examination was completed with the goal of clarifying until which extent each document refers the different studies categories as significant to be evaluated. To obtain such evidence the information coded at each node was analysed, allowing to compare the content of each document in relation to each sort of study.

The fact that all documents refer to the same non-clinical studies allowed to perform the aforementioned comparison between them, resulting in the information was resumed in table 2. Furthermore, to perform a clearer comparison between the diverse non-clinical data package, information available on each document will be compared in larger detail through the next sections, providing additional information to the one presented on the table below.

Table 2 - Summary table of requirements for non-clinical studies for each guideline considered

		SMALL MOLECULES	BIOTECHNOLOGY DERIVED	ATMPs	
		ICH M3(R2) [10]	ICH S6(R1) [11]	CELL-THERAPY (CHMP/410869/2006) [12]	GENE THERAPY (CAT/80183/2014) [13]
Pharmacology	Pharmacodynamics	Study mode of action; Drug's effects when compared to its intended therapeutic action	*In vitro* assays determining which effects of the product may be related to its clinical activity; In vitro cell lines to predict aspects of *in vivo* activity	Demonstrate proof-of-principle; In vitro studies to verify cell and tissue morphology, differentiation, proliferation, phenotype and heterogeneity; Determine best effective dose	Demonstrate proof-of-principle; *in vitro* and *in vivo* studies to unravel the mechanism of action; Determine best effective dose
Pharmacology	Safety Pharmacology	Study effects on major physiological systems	Study effects on major physiological systems	Assess if pharmacologically active substances, that cells may release, interact with major physiological systems	Investigate undesirable effects on major physiological systems
	Pharmacokinetics	Standard ADME studies	Standard ADME may not be useful; Metabolism studies not relevant; Evaluation of absorption, disposition and clearance	Standard ADME not relevant; Tissue distribution, viability, growth, differentiation, migration and phenotype	Standard ADME not relevant; Distribution, persistence, clearance and transcription of the administered nucleic acid; Germline transmission;
Toxicology	Single-dose studies	Acute toxicity studies; 2 mammalian species (clinical and parenteral routes of administration)	Dose-response relationship; Dose relation to systemic or local toxicity	Appropriate post-dose observation period, longer than standard pharmaceuticals' studies	Appropriate post-dose observation period, longer than for standard pharmaceuticals' studies
Toxicology	Multiple-dose studies	Up to 2 weeks use: one-month study; Up to 1 month: 3 months; 1 up to 3 months use: 6 months study; More than 3 months: 6 months rodent and 9 months non-rodent studies	Short-term use: studies up to 2 weeks; Chronic indications: 6 months studies	Only required if intended clinical use requires multiple dosing	Only required if intended clinical use requires multiple dosing or single dose results in prolonged function
	Local tolerance	As part of toxicity studies using the intended therapeutic route	As part of toxicity studies	Tissue compatibility and tolerance as part of toxicity studies	May be required as part of toxicity studies
	Genotoxicity	Gene mutation assay plus an additional assessment to detect chromosomal damage	Standard tests are not applicable	Not considered necessary except if product interacts directly with DNA	Assess genomic modifications and abnormal cell behavior; Identification of genomic integration sites
	Carcinogenicity/ Tumorgenicity	Only if there is significant cause for concern	Standard testes are generally not applicable; Only if product has potential to stimulate abnormal cell growth	Standard tests are not required; Study tumorigenesis (e.g. *in vitro*)	Standard tests are generally not applicable; in vivo/in vitro studies for neoplasm signals and cell proliferation
	Immunotoxicity	Standard studies for 28 days with consecutive daily dosing in a rodent	Detection of anti-drug antibodies; Study inflammatory response at injection site	Assess induction of immune response against cells or cell-derived products	Study of innate and adaptive immune response
	Reproduction studies	If is considered appropriate for the population to be exposed	If is considered appropriate for the population to be exposed	Case-by-case approach	Studies for germline transmission

4.1 Pharmacology

4.1.1. Pharmacodynamics

When comparing pharmacodynamic studies for small molecules, biopharmaceuticals and advanced therapy medicinal products, it was possible to comprehend that the core goal for these studies was the same, independently of the pharmaceutical type. In all the four guidelines analysed the same references were found, namely the objective of proof-of-principle study, as well as mode of action. Besides, in all the documents it was possible to find a reference to the conjugation of *in vitro* and *in vivo* data, using a relevant animal model and replicating the intended clinical use for adequate human extrapolation. Besides, also references to safe dose studies were encountered, with documents stating the importance of assessing dose selection for FIH trials.

One important topic to stand out is the absence of specific tests recommended to assess pharmacodynamics in the regulatory documents analysed. This is due to the diversity in pharmaceuticals' characteristics, that conducts to a diversity in PD tests to be performed. Therefore, not a single battery of tests is adequate for the different pharmaceuticals, even within the same category, such as small molecules, and so only the goal of this data acquisition is relevant to be included in the regulatory documents.

Furthermore, it was possible to find two distinct topics within pharmacodynamic studies, when considering the *Guideline on Human Cell-based Medicinal Products* [12], namely the primary pharmacodynamics and the secondary pharmacodynamics. The first one concern the effects that the pharmaceutical has in relation to its desired therapeutic effect. On the other hand, the secondary pharmacodynamic studies are related to substance's effects that are not correlated to the intended therapeutic outcome. This is of particular relevance for cell-based products considering that they might migrate and home at locations besides the intended one, leading to some undesired outcomes.

Differences were found particularly for ATMPs due to the necessity of performing *in vitro* studies to analyse cell and tissue morphology, differentiation and proliferation, as well as interaction with other tissues, when considering cell-based products. When studying gene therapy products, it is also important to understand if the nucleic acid sequence administered reaches the intended target and provides the proposed function. These requisites may not be relevant for small molecules being developed and for that reason are not referred in *ICH M3(R2).*

4.1.2 Safety Pharmacology

As well as it occurred with pharmacodynamic studies, also similarities were found in the safety pharmacology studies to be developed. Independently of pharmaceutical type, the main objective of these studies is to assess effects that the drug may incite in major physiological systems, particularly cardiovascular, respiratory, renal and central nervous systems. These studies may be performed separately or be assessed through the inclusion of relevant endpoints in toxicity studies or pharmacokinetic ones.

Differences remained with cell-based products where there is a necessity to study pharmacologically active substances released by the administered cell-based product, evaluating whether these substances also interact with major physiological systems.

4.2. Pharmacokinetics

This type of study, essential to support pharmacodynamic and safety data, was the one where it was possible to find key differences in each of the four scientific guidelines analysed.

For small molecules it is important to assess ADME, namely absorption, distribution, metabolism and excretion. On the other hand, for biopharmaceuticals, conventional ADME studies does not apply. Considering that biopharmaceuticals' metabolism is expected to lead to the degradation of small peptides and amino acids, metabolic pathways are generally well comprehended. Thus, conventional metabolism studies are not mandatory for these products. On the other hand, biodistribution, disposition and clearance studies have to be available for FIH studies.

When it comes to ATMPs, also standard ADME studies are not relevant. Data may be derived from specific pharmacokinetic studies or acquired through inclusion of relevant endpoints in other studies, such as toxicology. For cell-based products distribution, growth, viability, migration and differentiation are important to be assessed particularly due to cell migration capacity within the host from the site of administration. If cell-based products produce active substance it is also mandatory to assess these substance distribution and amount of expression. For gene therapy, the distribution, persistence, clearance and transcription of the administered nucleic acid sequence are important, as well as the study of germline transmission risk.

4.3. Toxicology

4.3.1. Single Dose Studies

Different studies are required according to the different pharmaceutical types considered. For small molecules, acute toxicology information is obtained from dose range finding studies or repeated dose studies. Physical effects and relevant biomarkers are monitored after drug's first administration, allowing to acquire relevant data with regards to acute toxicity. This leads to the absence of a separate single-dose studies for these pharmaceuticals. For biopharmaceuticals single-dose toxicity studies must allow determination of dose to systemic and local toxicity and no reference is made to specific requirements for such studies.

For ATMPs, both guidelines related to these pharmaceuticals state the necessity of toxicity studies with an appropriate post-administration observation period, longer than the ones required for small molecules. This is an important recommendation due to ATMPs' capacity of longer functional time

periods and induction of long-term effects. Furthermore, the route of administration and dose regimen should be similar to the proposed clinical one.

4.3.2. Repeated Dose Studies

Differences were also found when it comes to multiple-dose studies. For small molecules these studies must be performed for a time period longer than human clinical trial duration. In particular, for a treatment duration up to 2 weeks, a one-month study is considered appropriate. For treatments up to 1 month, a 3 months report is appropriate and for 1 up to 3 months, the study must be conducted for 6 months. All of them must be performed in a rodent and a non-rodent species. For treatments to be performed during more than 3 months, a 6 months rodent analysis and 9 months non-rodent one are adequate.

When it comes to biopharmaceuticals, the duration of these studies is also defined according to intended clinical duration and targeted group, taking as basis the recommendations available in *ICH M3(R2)* and adapting in accordance with biopharmaceuticals characteristics. In most cases, studies are performed during 1 to 3 months. For drugs used during less than 7 days, a study up to 2 weeks is acceptable and for pharmaceuticals designed for chronic treatment, a study of 6 months must be performed.

ATMPs differ in these requisites as repeated dose studies are only necessary if multiple dosing is proposed, which is not usual since ATMPs show a longer anticipated life-span, only requiring a single administration. An example that supports this recommendation is the unique administration of cell-based products, which leads to the absence of these studies' requirement. For gene therapy products repeated dose analyses are also conducted only if long pharmaceuticals' functional periods are expected.

4.4. Local Tolerance

With regards to local tolerance studies the four documents analysed refer to the same possibility of including local tolerance studies as part of other general toxicity studies. For standard molecules, it is important to use the intended route of administration, since different routes can lead to a different response to the drug being tested.

When it comes to ATMPs evaluation these tests are not seen as mandatory and may be performed according to the type of pharmaceutical, its mode of action and route of administration.

Thus, even for small molecules and biopharmaceuticals for which local tolerance must be assessed it is adequate to include them in other studies and consequently, avoid dedicated local tolerance tests.

4.5. Genotoxicity

When it comes to genotoxicity it is possible to see great differences in recommendations for different pharmaceuticals, taking into consideration that it may not be relevant to perform such tests for some medicinal products.

For small molecules, a gene mutation assay is recommended to be performed and if there is any additional sign of concern an assay to detect chromosomal damage must be implemented.

On the other hand, the recommendations for biopharmaceuticals state that standard tests, as the ones performed for small molecules, are not relevant for this type of drugs, since it is not expected that these substances are capable of interact with genetic material or lead to any chromosomal alteration.

Considering ATMPs, there are great differences in genotoxicity recommendations since cell-based therapies and gene therapies face crucial dissimilarities in their mode of action and consequently in the interaction with genetic material. For cell-based products genotoxicity tests are not seen as relevant since these products are not expected to interact with DNA directly, as occurs with biopharmaceuticals. For gene therapy products genotoxicity tests are considered relevant to assess possible genomic modifications and subsequent altered cell behaviour, assess outcomes of insertional mutagenesis and identify locals of genomic integration.

4.6. Carcinogenicity/Tumorigenicity

For small molecules, according to *ICH M3(R2)* [10], standard carcinogenicity studies must be performed if they are considered relevant for the proposed clinical indication, particularly if they are intended to be used for a period longer than 6 months or in an intermittent and repeated manner. Other cause for concern is if the pharmaceutical was proved to be genotoxic, leading to the necessity of performing long-term studies, due to possible hazards that these pharmaceuticals may present.

In the other three regulatory guidelines it is stated that standard carcinogenicity studies, as the ones conducted for small molecules, are not relevant for biopharmaceuticals or ATMPs. Independently of the novel medicinal product under evaluation, these studies must be performed for at least 6 months. For biopharmaceuticals such evaluation must be performed if the product is expected or has been proved to show great potential to incite abnormal cell growth. If so, it is relevant to assess receptor expression in different human cells. When considering cell-based products, tumorigenesis studies are of major relevance since there may exist a very high probability of neoplastic alterations induction in cells contained in the pharmaceutical or in host cells. This can be justified by the fact that these cell-based products present migration, differentiation and proliferation capacity, not seen in standard pharmaceuticals. To assess tumorigenic potential of CBMP, *in vitro* tests must be performed, analysing tissue and/or cells' behaviour and acquiring data on proliferation, differentiation and cell-derived products. When it comes to gene therapy products it is also relevant to perform such studies of tumorigenic potential to assess neoplastic signals and oncogene activation.

4.7. Immunotoxicity

These tests are considered relevant for most pharmaceuticals development programs since one of the major issues with a new drug's administration is the human inflammatory response that may develop afterwards, induced by the immune system.

Small molecules' studies must be performed in a rodent for 28 consecutive days of daily medicinal product's administration, as explored in larger detail in *ICH S8 – Immunotoxicity Studies for Human Pharmaceuticals* [51]. When it comes to biopharmaceuticals, many of them are intended to supress or stimulate the immune response, representing the therapy's target. Therefore, immunotoxicity is verified when there are nontarget immune effects, such as autoimmunity. To conduct immunotoxicological evaluation, antibody response must be described when performing single- and/or repeated dose studies. In case of relevant evidence found, additional immunotoxicology testing may be based on screening studies followed by mechanistic ones.

For cell-based products it is considered relevant to assess possible immune response against cells included in the product or active substances derived from the pharmaceuticals under assessment. Finally, for gene therapy products the administration can lead to an activation of adaptive and innate immune responses, and so it is important to consider aspects that can influence this outcome such as gene transfer protocols, transgene transport vehicle and transgene product.

4.8. Reproduction Toxicity

When it comes to reproduction toxicity studies or development studies, different scientific documents show similarities. It is stated for small molecules, biopharmaceuticals or cell-based products that these studies must be performed according to the intended exposed population and expected pharmaceutical's mode of action. When studying small molecules, specific situations are explored, so it is possible to encounter differences according to the targeted population. Thus, reproduction studies are performed for men and women not of childbearing potential if during repeated dose toxicity studies relevant information on this topic was encountered. On the other hand, if women of childbearing potential are to be included in the trial it is important to assess unintentional exposure of an embryo or foetus. Furthermore, if pregnant women belong to the targeted population, female reproduction toxicity studies must be performed, as explored in *ICH S5(R2) – Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility* [52], as well as genotoxicity evaluation. Therefore, it is possible to understand that reproduction outcomes must be assessed in a case-by-case approach.

Particular relevance of these studies can be found when developing gene therapy medicinal products, where it is important to assess effects on general reproductive function and fertility. Furthermore, for these pharmaceuticals it is important to comprehend the risk for germline transmission and so the possibility of nucleic acid transmission to the offspring must be studied.

4.9. Conclusions on preclinical clinical data package

Consequently, from the results obtained with NVivo for the different guidelines, it was possible to clarify that every document analysed referred to each one of the non-clinical studies considered. Afterwards, the detailed comparison conducted for each preclinical study type, allowed to appreciate that references to each test occur even if they were considered not relevant for the pharmaceutical under study, according to its characteristics and a justification on the decision of not performing a certain non-clinical study type must be provided. Besides, it was possible to comprehend that although each guideline gives specific recommendations with regards to the pharmaceutical that is being considered, there is still similarities between different medicinal products non-clinical evaluation. These similarities are bigger when looking at each test purpose, which is justified by the fact that the core goal of preclinical studies is the same, independently of which pharmaceutical is being evaluated and differences are mainly encountered in the way data is obtained for each medicinal product.

5.Case Studies analysis

Given the focus of this thesis on cell-based medicinal products, the case studies selected belong to somatic cell therapies and tissue engineered products, bearing in mind the methodology explored in section 2.4 of the present document. From this first constrain applied, the ATMPs that remained are depicted in table 3.

Table 3 - Overview of ATMPs evaluated by EMA and selected as relevant for this analysis

ATMP name	ATMP classification	Therapeutic indication	Pharmacotherapeutic group
ChrondroCelect [53]	TEP	Cartilage Defects	Disorders of the musculo skeletal system
MACI [54]	TEP	Cartilage Defects	Disorders of the musculo skeletal system
Provenge [55]	sCTMP	Prostatic Neoplasms	Immunostimulants
Holoclar [56]	TEP	Corneal diseases	Ophthalmologicals
Zalmoxis [57]	sCTMP	Graft versus Host Disease	Antineoplastic agents
Spherox [58]	TEP	Cartilage Defects	Disorders of the musculo skeletal system
Alofisel [59]	sCTMP	Rectal Fistula	Immunosupressants
Heparesc [60]	sCTMP	Urea Cycle Disorders	–*1

*1 It was not possible to found information related to Heparesc's pharmacotherapeutic group due its marketing authorization refusal.

Considering the similarities and differences, possible to encounter after analysing these products' assessment reports, it was considered relevant to perform separate studies.

The first evaluation performed, explored in section 5.1, gathers conclusions and results obtained after comparison of ATMPs' assessment reports belonging to the same pharmacotherapeutic group, amending the same therapeutic indication and showing a similar mode of action and administration. When it comes to the second analysis conducted, discussed throughout section 5.2, ATMPs were classified using a new classification system, created considering products' mode of action and risks inherent to their use. Furthermore, this section bears in mind the risk-based approach and evaluates its influence in preclinical studies analysed. Finally, in section 5.3, non-clinical testing properties shared between considered ATMPs are overviewed and discussed, considering the recommendations available on the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16].

Firstly, it is important to stand out that from the ATMPs present in the table above, two of them were not considered for further review, namely Holoclar and Provenge. Holoclar was not considered for further case-studies analysis since it showed a very distinct therapeutic indication from the other three

tissue-engineered products considered and the product non-clinical development program was not considered relevant to be analysed. Besides, also Provenge was not considered since it intended to show a therapeutic outcome on prostatic neoplasms and the oncology field was not explored in this study, thus this medicinal product was not considered for additional analysis.

Before further discussion, a brief overview of the ATMPs' selected for further discussion will be provided, crucial to have a clearer image of the products' characteristics and easily comprehend the preclinical studies performed for each of these medicinal products.

ChrondroCelect [61]

ChrondroCelect, developed by TiGenix, was granted a European Marketing Authorization in 2009, being the first ATMP approved by the Committee for Medicinal Products for Human Use (CHMP). Classified as a tissue-engineered product, it is cell-based, being formed by autologous chondrocytes expanded *ex vivo,* and delivered as a suspension, to treat symptomatic cartilage defects. This medicinal product is intended to be administered as part of a two-step procedure. The first step corresponds to a cartilage biopsy, during which, healthy cartilage tissue is extracted from patient's knee. Thereafter, cartilage cells are expanded *ex vivo* and characterised by specific marker proteins. During treatment's second stage, the suspension containing the chondrocytes is administered to the same patient in an open-knee surgery, being placed into knee's defect and kept in place by a biological membrane. Intended purpose of this procedure is the formation of new hyaline cartilage and consequent treatment of cartilage defects from 1 to 5 cm^2 size.

Since 2016 ChrondroCelect was withdrawn from the market due to commercial reasons [62]. Furthermore, it is relevant to stand out that ChrondroCelect had pre-authorisation use, which might alter the non-clinical studies' design, since some evidence might have been acquired from pre-authorisation use.

MACI [63]

MACI, from Genzyme, was available throughout the European market since 2013. As well as ChrondroCelect, this tissue-engineered product is intended to show a therapeutic effect on symptomatic cartilage defects with a size that can range from 3 to 20 cm^2. It consists of patient's own chondrocytes applied to a porcine derived collagen matrix (ACI-Maix matrix), which is held in place using a fibrin glue. MACI is intended to regenerate articular tissue and restore knee's function after insertion of autologous cartilage cells onto the defect site, using a two-step procedure to firstly obtain the patient's own cells, expand it *ex vivo* and lastly place it onto cartilage defect.

Since 2014 MACI is no longer available in the market, due to lack of an authorised European manufacturing site, which caused the expiry of its marketing authorisation [64].

Spherox [65]

Spherox, such as ChrondroCelect and MACI, intends to repair symptomatic cartilage defects of the knee, allowing to restore knee's functionality. Cartilage defects' size must be up to 10cm^2. This tissue-engineered product was developed by CO.DON, and received a European marketing authorisation in 2017, being still available on the market. It consists of 3-dimensional structures, namely spheroids, containing human autologous chondrocytes associated to a self-synthesized matrix. Spherox is developed and administered to the patient in a two-step process. Firstly, patient's articular cells are extracted during a cartilage biopsy. Thereafter, cells are expanded, and spheroids are formed. Finally, the final product is delivered to the patient as a suspension containing cell aggregates.

Alofisel [66]

Alofisel, from TiGenix, is an allogeneic somatic cell therapy, developed to have a therapeutic action on complex perianal fistulas in Chron's disease patients. This ATMP has granted a European marketing authorization in 2017 and is still available on the market, being the most recent cell therapy approved within the ATMPs considered to conduct this study. It consists of expanded human allogeneic adipose stem cells delivered as a suspension in a single administration. This expanded stem cells are intended to modulate immune response and show anti-inflammatory properties, such as the inhibition of pro-inflammatory cytokines release. This therapeutic action aims to reduce local inflammatory signs and consequently allow tissues in the perianal fistula's area to heal.

Heparesc [67]

Heparesc, developed by Cytonet GmbH & Co KG, intended to show a therapeutic action on urea cycle disorders. It was classified as an allogeneic somatic cell therapy proposed to be used in children until they have all the essential health parameters to receive a liver transplant. This medicinal product consisted of human heterologous liver cells, intravenously infused through an intraportal catheter, in six daily doses. Liver cells present in Heparesc were acquired from non-transplantable donor organs, that are capable of producing the enzyme that the patient's liver is not. Cells infused were expected to integrate into patient's liver tissue after administration via portal vein bloodstream, allowing to produce the missing enzyme, thus reducing disease symptoms.

This ATMP has not been granted a European marketing authorization due to clinical studies developed that did not allowed to obtain adequately successful results as required. Nevertheless, it was considered relevant to study its non-clinical assessments, since they were considered adequate to begin with first clinical studies [68].

Zalmoxis [69]

Zalmoxis, approved since 2016 and developed by MolMed, intends to be used as an adjunctive therapy after hematopoietic stem cell transplantation from a partially matched donor (haploidentical). The aim of this ATMP is to support immune system reconstruction, allowing to control opportunistic

infections that might occur, while the host immune system is undergoing hematological reconstruction, after an haploidentical hematopoietic stem cell transplantation (HSCT). Furthermore, this cell-therapy mechanism of action allows to avoid possible adverse events, particularly Graft vs. Host disease and help fight cancer that might develop, due to its suicide gene mechanism. For this somatic cell therapy, donor T cells are collected, isolated and engineered to include a suicide gene, which is activated, by antiviral drugs, if the patient shows some signs of adverse reaction after the transplant allowing to selectively eliminate dividing cells, the alloreactive T cells. Thus, the host shows different types of T cells, the ones derived from hematopoietic stem cells, received during transplant and T cells included in Zalmoxis. This therapy is delivered as an infusion in a single monthly dose, up to four months, to patients who transplant for itself was not capable of restore the immune system and who show high risk of hematological malignancies. Zalmoxis is still available on the market and received a European marketing authorization in 2016.

Furthermore, the analysis of medicinal products' assessment reports allowed to acquire information related to non-clinical studies, which was summarised for each medicinal product and are present in the tables depicted next. These tables show data related to studies' duration and animal models used, as well as a brief description of main goals of each study performed and how they were conducted in order to acquire the intended non-clinical data. Thus, table 4, 5, 6, 7, 8 and 9 summarises ChondroCelect, MACI, Spherox, Alofisel, Heparesc and Zalmoxis non-clinical data packages, respectively.

Table 4 - Summary of non-clinical studies conducted for ChrondroCelect [70] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
ChrondroCelect	Pharmacology	Primary Pharmacodynamics	Comparison of intramuscular injection of human articular chondrocytes vs. normal human cartilage	2 W	Mice
			Comparison between late passage and early passage chondrocytes implantation in cartilage formation	N.S	
			Assessment of repair efficacy in the defect center and repair tissue integration between differentiated and dedifferentiated cells	N.S	Goat
			Comparison between repair capacity using passage 1 vs passage 5 chondrocytes	N.S	
			Assessment of mobility and filling of cartilage lesion between hyaline-like cartilage and hyaline fibrocartilage	53 W	
		Secondary Pharmacodynamics	Not performed	-	-
		Safety Pharmacology	Not performed	-	-
	Pharmacokinetics	Biodistribution	Assess cells' migration capacity from implantation site and consequent persistence in the cartilage defect, using fluorescently-tagged chondrocytes	N.S	Goat
	Toxicology	Single-Dose	Assess intramuscular or subcutaneous injection of different cell types (pig, goat, human) (1)	12 W	Mice
			Evaluate cells penetration in subchondral bone (1)	14 W	Sheep
			Assess autologous vs. human chondrocytes implantation outcomes such as inflammation, bone or ectopic cartilage formation, through macroscopic, histological and biochemical analysis of synovial fluid and synovium (1)	10 W, 52 W	Goat
		Repeated dose	Not performed	-	-
	Genotoxicity		Not performed	-	-
	Carcinogenicity/Tumorigenicity		Assess human chondrocytes senescence after several passages and consequent risk of tumorigenic growth	N.S	*In vitro*
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Assess local response during toxicology studies (1)	-	Mice
			Assess local response in orthotopic models during toxicology studies (1)	-	Goat
			Assess local response in orthotopic models during toxicology studies (1)	-	Sheep

(1) Local tolerance assessed during toxicological studies

Table 5 - Summary of non-clinical studies conducted for MACI [71] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
MACI	Pharmacology	Primary Pharmacodynamics	Evaluation of MACI's efficacy, analyzing cartilage repair with MF and no-MF procedure	12, 24 W	Rabbit
			MACI implant vs. untreated defect; Assessment of percent filling, surface smoothness, peripheral and basal integration, tissue color and softness; Biochemical composition of repair tissue evaluated at 26 weeks' time-point (1)	13,26 W	Horse
			Assess long-term performance; Group1: MACI vs. cell-free membrane; Group 2: MACI vs. untreated defect; Same parameters analysed for previous study; Blood's characterization, synovial tissue biopsy, mechanical testing and cartilage's histologic and immunohistochemistry evaluation (collagen II formation) and chondrocyte predominance (2)	53 W	Horse
		Secondary Pharmacodynamics	Assess rabbit cells viability before seeding to ACI-Maix membrane and after in three different time points; Evaluation of cells-membrane interactions	4, 7 D	*In vitro*
			Assess human cells viability after seeding to ACI-Maix membrane; Evaluation of chondrocytes adhesion	3, 14 D	*In vitro*
		Safety Pharmacology	Assess effects on major physiological systems; Hematological/serum and synovial fluid analysis (2)	53 W	Horse
	Pharmacokinetics	Biodistribution	Assess cells' migration capacity from implantation site; Histological evaluation of chondrocytes' existence in different lymph nodes; Assessment of ectopic cartilage tissue formation (2)	53 W	Horse
	Toxicology	Single-Dose	Intravenous and intraperitoneal injection; ACI-Maix collagen matrix evaluation for toxicity outcomes in different organs (heart, lungs, liver, spleen, etc.)	4, 24, 48, 72H	Mice
			Assess MACI efficacy; Evaluation of blood counts, synovial fluid and synovial inflammation (1)	13, 26 W	Horse
			Assessment of blood characterization, synovial tissue, effects on major organ systems, serum chemistry, synovial membrane histology (2)	53 W	Horse
		Repeated dose	Not performed	-	-
	Genotoxicity		Not performed; Only mutagenicity studies to assess interaction with chromosomal material; Evaluation of cells karyotype at different culture time-points	N.S	*In vitro*
	Carcinogenicity/Tumorigenicity		Not performed	-	-
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Assessment of local tolerance to ACI-Maix membrane; (*)	4, 13 W	Rat
			Assessment of ACI-Maix membrane irritation potential after muscle implantation	N.S	Rabbit
			Assess toxic effects that may occur; Evaluation of local tolerance persistence over time; Evaluation of membrane degradation; Assessment of foreign body response, necrosis or hemorrhage	12, 26 W	Rat
			Assess potential for irritation due to ACI-Maix membrane extracts after intracutaneous injection	N.S	Rabbit
			Assess synovial tissue alterations due to inflammation (1)	13, 26 W	Horse
			Assess synovial tissue alterations due to inflammation (2)	53 W	Horse

(*) – Two different studies performed, with the same goal and same animal models used and so they were merged as a single one with two different time points

MF – Microfracture

(1) Same study performed, assessing pharmacodynamics, single-dose toxicity and local tolerance

(2) Same study performed for pharmacology, biodistribution and local tolerance

Table 6 - Summary of non-clinical studies conducted for Spherox [72] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
Spherox	Pharmacology	Primary Pharmacodynamics	Evaluation of spheroids formation by human chondrocytes; Assessment of spheroid size and viability (necrosis and apoptosis); Evaluation of chondrocyte differentiation markers and assessment of hyaline ECM formation; Hyaline specific markers were used (collagen type II and aggrecan) (1)	8 W	*In vitro*
			Assessment of adhesion, fusion and integration of spheroids to host tissue; Spheroids and cartilage explants cultured to assess adhesion to explants' surface, chondrocytes migration and cell-cell/matrix interactions (2)	11 W	*In vitro*
			Assessment of proof of principle (regeneration of hyaline cartilage tissue); Human spheroids cultivated on cartilage explants and subcutaneously implanted in immunocompromised mice; Evaluation of integration, remodeling and migration of spheroids; Evaluation of *de novo* repair tissue formation	N.S	Mice
			Porcine spheroids used to assess integration and defect filling; Evaluation of repair tissue properties (histological/immunohistological studies and biomechanical properties) (4)	8 W	Minipig
			Autologous ovine spheroids used to evaluate repair tissue formation (5)	26, 39 W	Sheep
		Secondary Pharmacodynamics	Not performed	-	-
		Safety Pharmacology	Not performed	-	-
	Pharmacokinetics	Biodistribution	Assess local and systemic distribution of cells; Analysis of lymph nodes, liver, lungs, spleen, etc. (3)	4 W	Mice
			Assess spheroid adhesion; Fluorescently labelled spheroids used to study local biodistribution	N.S	Sheep
	Toxicology	Single-Dose	Safety was evaluated with regards to cell senescence and proliferation (1,2)	N.S	In vitro
			Single dose injected subcutaneously evaluating safety endpoints (3)	-	Mice
			Single dose injected into the knee joint (4)	-	Minipig
			Single dose injected to assess systemic toxicity after 6 months in a condyle defect (5)	26 W	Sheep
		Repeated dose	Not performed	-	-
	Genotoxicity		Not performed	-	-
	Carcinogenicity/Tumorigenicity		Evaluate ectopic tissue or tumor formation; Evaluation of tumorigenic and migration potential from administration site (3)	26 W	Mice
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Evaluation of toxicity outcomes due to autologous spheroids implantation; Assessment of possible adverse effects and tolerability to implanted spheroids (5)	26 W	Sheep

(3) Same study performed, assessing pharmacodynamic parameters and single-dose toxicity evaluations and single dose toxicity
(4) Same study performed assessing pharmacodynamics and single-dose toxicity single-dose toxicity and local tolerance
(5) Same study evaluating pharmacokinetics, toxicity and carcinogenicity
(4) Same study performed for proof-of-concept
(5). Same study performed for pharmacodynamics,

Table 7 - Summary of non-clinical studies conducted for Alofisel [73] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
Alofisel	Pharmacology	Primary Pharmacodynamics	Intraperitoneal administration of eASC to assess overall survival, dose dependence body weight and levels of immunological activity	N.S	Mice
			Assess function of eASC in induction of Treg cells by administration of T cells from eASC pre-treated mice and no pre-treated mice	N.S	Mice
			Dose dependence and clearance studied by intravenous infusions, with observational test battery (motor activity, behavioral parameters and reflex responses)	2, 26 W	Mice
		Secondary Pharmacodynamics	Not performed	-	-
		Safety Pharmacology	CNS evaluation; Functional test battery (motor activity, behavioral parameters, coordination and reflex responses) performed to assess outcomes with different dose levels; Study performed with two intravenous administrations with a 2-week interval	2, 26 W	Mice
	Pharmacokinetics	Biodistribution	Assessment of cells migration using perianal and intrarectal route; Evaluation of migration to different organs (heart, liver, kidney, jejunum, ileum, rectum, uterus) by detection of human DNA	1 D, 14 D 6 W, 12 W, 26 W	Mice
			Assessment of cells migration when using intravenous route	1 D, 14 D 6 W, 12 W, 26 W	
			Assessment of cells migration when using intravaginal route	1D, 14D 6W, 12W, 26W	
	Toxicology	Single-Dose	Evaluation of systemic toxicity and local inflammatory alterations when using intravenous route and different doses by microscopic observation	14 D	Mice
			Evaluation of systemic toxicity and local inflammatory alterations when using subcutaneous route and different doses by microscopic observation	14 D	Mice
		Repeated dose	Evaluation of dose dependent systemic toxicity; 2 doses administered with 2-week interval using perianal route	12 W	Mice
			Evaluation of dose dependent systemic toxicity; 2 doses administered with 2-week interval using perianal route	24 W	
			Evaluation of dose dependent systemic toxicity; 2 doses administered with 2-week interval using intravenous route	26 W	
	Genotoxicity		Not performed	-	-
	Carcinogenicity/Tumorigenicity		Assessment of cell growth (population doubling level), occurrence of cells' senescence, telomerase activity and anchorage-independent growth	N.S	*In vitro*
			Subcutaneous injection of eASC used to evaluate tumor formation at different population doubling levels	N.S	Mice
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Performed as part of single and repeated dose studies	-	-

Table 8 - Summary of non-clinical studies conducted for Zalmoxis [74] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
Zalmoxis	Pharmacology	Primary Pharmacodynamics	Phenotypic analysis of MM-TK, evaluating expression of CD3, CD4, CD8 and activation marker such as CD69 and CD25	N.S	*In vitro*
			Evaluation of cytokine production to assess cell transduction to Th1 and Th2	N.S	*In vitro*
			Response to intended medicine to be used (ganciclovir) evaluated as part of quality control studies	N.S	*In vitro*
			MM-TK cell engraftment, long-term safety and efficacy of the proposed suicide system are evaluated with mice being treated with GCV or a negative control; Evaluations performed based on body weight, chimerism and GvHD	N.S	Mice
			Evaluation of alloreactive responses based on a subcutaneous transplant of human skin; Evaluations performed based on body weight, chimerism and GvHD	N.S	Mice
		Secondary Pharmacodynamics	Not performed	-	-
		Safety Pharmacology	Not performed	-	-
	Pharmacokinetics	Biodistribution	MM-TK cells traced with a focus on lymphohaematopoietic and non-lymphohaematopoietic organs, assessing human T cell infiltration; Tissues studied by histology and immunohistochemical assessment using anti-human CD3mAb to evaluate human T cells presence (by CD3+)	N.S	Mice
	Toxicology	Single-Dose	Not performed	-	-
		Repeated dose	Not performed	-	-
	Genotoxicity		Not performed	-	-
	Carcinogenicity/Tumorigenicity		Assessment of MM-TK oncogenic risk; Evaluation of clonality of the transduced cell population; Characterization of the insertional pattern; Evaluation of cells survival dependence on growth factors	N.S	*In vitro*
			Correlation of expression level with the ability of retroviral vectors to integrate in particular sites, by microarray analysis; T cell gene expression profile, phenotype and biological function assessment	N.S	*In vitro*
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Not performed	-	-

Table 9 - Summary of non-clinical studies conducted for Heparesc [67] (N.S – Not specified)

Name	Type of non-clinical study	Subtype of non-clinical study	Goal and method	Duration	Animal model(s)
Heparesc	Pharmacology	Primary Pharmacodynamics	Proof-of-concept studies using rabbit liver cells preparations; Evaluation of LDL cholesterol levels in blood; Study the concept of intraportal hepatocytes perfusion	N.S	Rabbit
		Secondary Pharmacodynamics	Not performed	-	-
		Safety Pharmacology	Not performed	-	-
	Pharmacokinetics	Biodistribution	Evaluation of sinusoidal uptake capacity, pulmonary shunting, portal hemodynamic alterations and distribution to other body sites after intraportal administration	N.S	Rabbit
	Toxicology	Single-Dose	Not performed (2)	-	-
		Repeated dose	Evaluate the viability of the implementation procedure, with implantation of portal vein catheters through subcutaneous access; Study feasibility of liver cells administration into portal vein using the intended process, using different cell doses	-	Rabbit
			Evaluate toxicity of allogeneic liver cells using rabbit liver cells; Study local and systemic signs of inflammatory process, as well as portal hypertension due to administration process, lung shunting and immunological reactions (1)	-	Rabbit
	Genotoxicity		Not performed	-	-
	Carcinogenicity/Tumorigenicity		Not performed	-	-
	Reproduction Toxicity		Not performed	-	-
	Local Tolerance		Evaluate local post-administration symptoms such as fibrosis (1)	N.S	Rabbit

(1) Local tolerance study performed as part of repeated dose ones, with local evaluation of procedure outcomes
(2) Single-dose studies were not performed to evaluate the final product. Only the buffering agent (HEPES) was evaluated to assess possible toxic reactions due to its use as part of the final medicinal product.

5.1. Chondrocyte-based medicinal products

In this first section, results will be explored with regard to some specific features of medicinal products' study design, such as objectives of each non-clinical study conducted, animal model used and duration of each evaluation. For this analysis to be significant it is important that medicinal products considered can be compared when it comes to various characteristics such as mode of action and intended therapeutic outcome.

Thus, from the ATMPs present in table 3, it was considered meaningful to compare development processes of the medicinal products with the same ATMP classification, belonging to the same pharmacotherapeutic group and showing the same therapeutic indication, allowing to assess specific and meaningful similarities and differences with regard to particular aspects of studies' design, such as animal models used and each study duration. Thus, considering ATMPs classification, products' therapeutic indications, pharmacotherapeutic groups and modes of action, it was possible to understand that ChrondroCelect, MACI and Spherox amended a similar therapeutic indication and have a comparable mode of action, making them good candidates for further appraisal. Therefore, considering ChrondroCelect, MACI and Spherox characteristics, previously summarised, these ATMPs can be classified as chondrocyte-based medicinal products, intended to repair knee's structure and function.

An overall view of the methodological process used to obtain this comparative analysis results was previously described in section *2.4.1 Comparison of selected case studies' EPARs.* The results obtained in this first stage are similar to the ones present in chapter *4. Preclinical Data package supporting FIH.*

Thus, from the analysis performed with the software NVivo it was possible to have a clearer picture of performed studies for each medicinal product considered. Similar to what occurred in section 4, a matrix illustrating the relation between different products' assessment reports and non-clinical studies categories was obtained. However, it is important to stand out that this matrix was not obtained with the exact same coding strategy as the previous one. In the previously obtained matrix, the number 1 or 0 determined if there was any reference to the studies evaluated, even if, after guidelines' analysis, the studies coded with a specific node were not considered relevant. For example, with regards to genotoxicity, although biopharmaceuticals guideline referred this study, it was not considered relevant for that type of medicinal products.

On the other hand, for the present study phase, the matrix does not assess the reference to each study, because all EPAR documents must refer to all study types. Instead, it shows the performance and relevance of each study category for the considered medicinal products. Thus, matrix present in this section is not expected to show all entrances as 1, since products' features do not justify the implementation of all studies. The obtained matrix is present below.

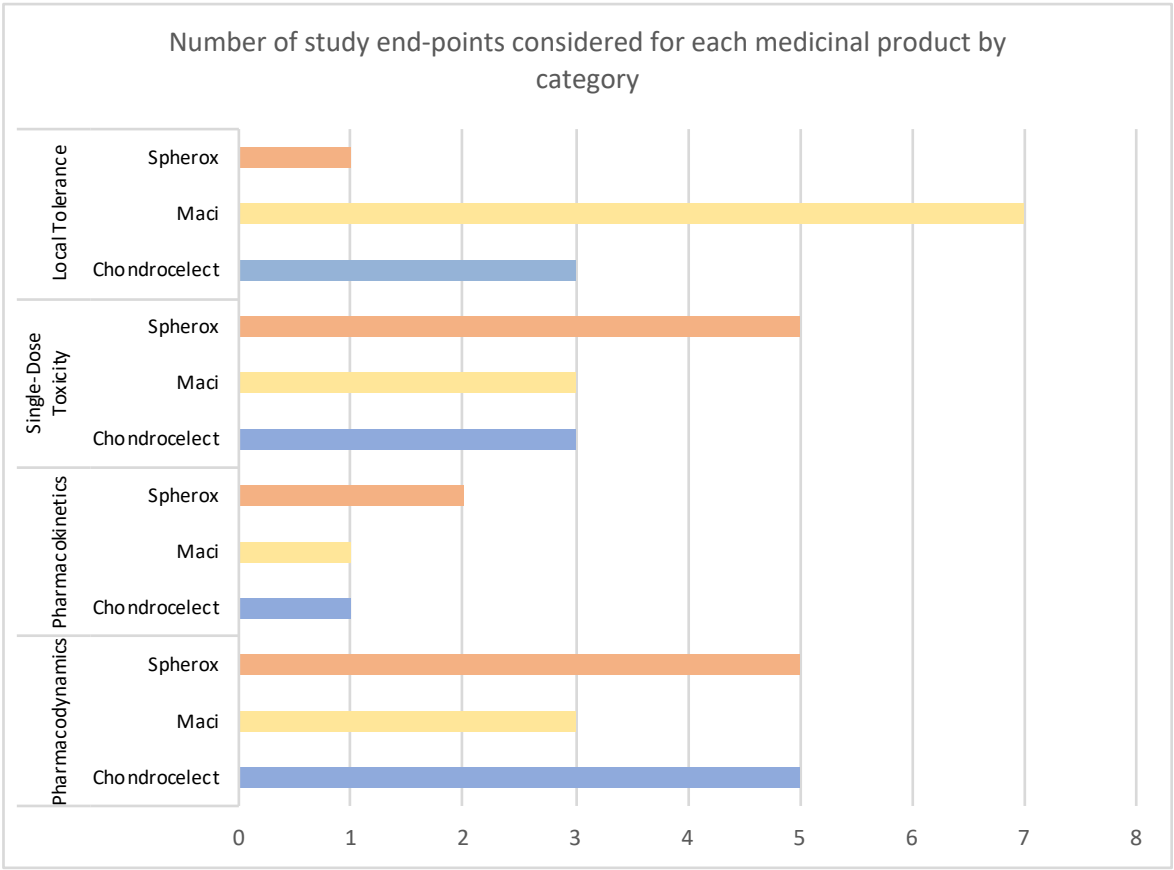
Table 10 - Non-clinical studies performed for chondrocyte-based medicinal products

	A: ChrondroCelect	B: MACI	C: Spherox
1: Primary Pharmacodynamics	1	1	1
2: Secondary Pharmacodynamics	0	1	0
3: Safety Pharmacology	0	1	0
4: Pharmacokinetics	1	1	1
5: Single-dose Toxicity Studies	1	1	1
6: Multiple Dose Toxicity Studies	0	0	0
7: Genotoxicity	0	0	0
8: Carcinogenicity/Tumorigenesis	1	0	1
9: Reproduction Toxicity	0	0	0
10: Local Tolerance	1	1	1

Moreover, it is relevant to note the absence of immunotoxicity studies for this comparison since this topic is not discussed as a separated study category in any of the case studies selected and so it was not included as a node in the analysis performed with NVivo. The medicinal product' EPAR that name immunotoxicity is ChrondroCelect but only includes it as part of other toxicity studies. The absence of this type of non-clinical studies is considered appropriate since none of the three chondrocyte-based medicinal products are expected to influence the immune system and develop some immunotoxicity.

Since number 1 illustrates the performance of a specific study and number 0 the absence of it, it is possible to understand, from table 10 analysis, that some similarities and differences can be found between the selected medicinal products. When it comes to repeated dose toxicity, genotoxicity and reproduction toxicity, no applicant performed such studies, considering them not relevant bearing in mind the products' features and mode of action. On the other hand, some studies were conducted in all three medicinal products' development processes, namely primary pharmacodynamics, pharmacokinetics and local tolerance.

Furthermore, the information collected was crucial to develop a statistical analysis particularly with regard to the number of studies performed, the animal models used for each medicinal product's development process and the duration of the different studies, depicted in Figure 8, Table 11 and Figure 9, respectively. Conclusions obtained from these results' analyses will be explored in the following sections, along with each non-clinical study analysis.



From the analysis of figure 8 it is possible to assess that the number of studies performed for each category vary according to the chondrocyte-based medicinal product considered, due to their characteristics and anticipated effects. One of the most important information available in this graph is the higher number of local tolerance studies performed for MACI. This occurrence can be justified by the presence of an external matrix as part of this medicinal product, which makes it relevant to assure as much as possible, the safety of the administered matrix. Furthermore, it is relevant to stand out the lower number of biodistribution studies performed for the three considered ATMPs since they are expected to have a local therapeutic effect and are intended to be administered locally, so no significant migration from implantation site is expected, however it is still assessed for the three ATMPs. Moreover, no other study categories verify relevant similarities or differences and a more detailed analysis will be conducted next, when discussing in more detail the studies performed for each non-clinical evaluation.

Table 11 - Animal models used for chondrocyte-based medicinal products

Medicinal Products	Mouse	Rabbit	Minipig	Sheep	Goat	Horse
ChrondroCelect	✓✓✓ (N.S)	-	-	✓(N.S)	✓✓✓✓ ✓ (N.S)	-
MACI*	✓✓✓(N.S)	✓✓✓ (20;N.S;N.S)	-	-	-	✓✓(6;27)
Spherox	✓✓ (N.S)	-	✓(5)	✓(N.S;3)	-	-

*When it comes to MACI it is important to stand out that the information present in this table is only related to studies performed by the applicant and it does not give respect to previously performed studies. Studies not conducted by the applicant used two equal species, namely horse and rabbit, and a third one not used in the applicant's study, the sheep.

From the table present above, it is easy to comprehend that similar animal models were used to perform non-clinical studies in chondrocyte-based medicinal products. Particularly all ATMPs used mouse animal models to perform initial *in vivo* studies. Furthermore, it is easy to assess the use of larger animal models for these ATMPs, relevant to perform functional studies and better mimic human physiological and anatomical features. Thus, to evaluate therapeutic effect in recovering knee's normal function, larger animal models were used. Although these models are not the same for all assessed ATMPs the relevant point is the use of larger species for all non-clinical studies performed for chondrocyte-based medicinal products, particularly goat for ChrondroCelect, horse for MACI and sheep for Spherox. Further discussion of this conclusions will be presented throughout next sections.

Lastly, the duration of non-clinical studies was also evaluated for the three chondrocyte-based medicinal products and the results of such evaluation are depicted in the next figure, figure 9. From the analysis of the graph available, it is crucial to stand out the conduction of long-term studies during 52, 53 and 39 weeks, allowing to assess long-term effects that might be expected upon human administration. Such evaluation is relevant since these three medicinal products also intend to have long-term therapeutic effects when administered to patients, so it is required that they show good safety and efficacy profiles when conducting long-term evaluations *in vivo*. Further discussion on each study duration will be conducted next.

The brief statistical analysis performed serve as basis for easily compare the studies performed for ChrondroCelect, MACI and Spherox and are intended to be considered in conjunction with next section reading.

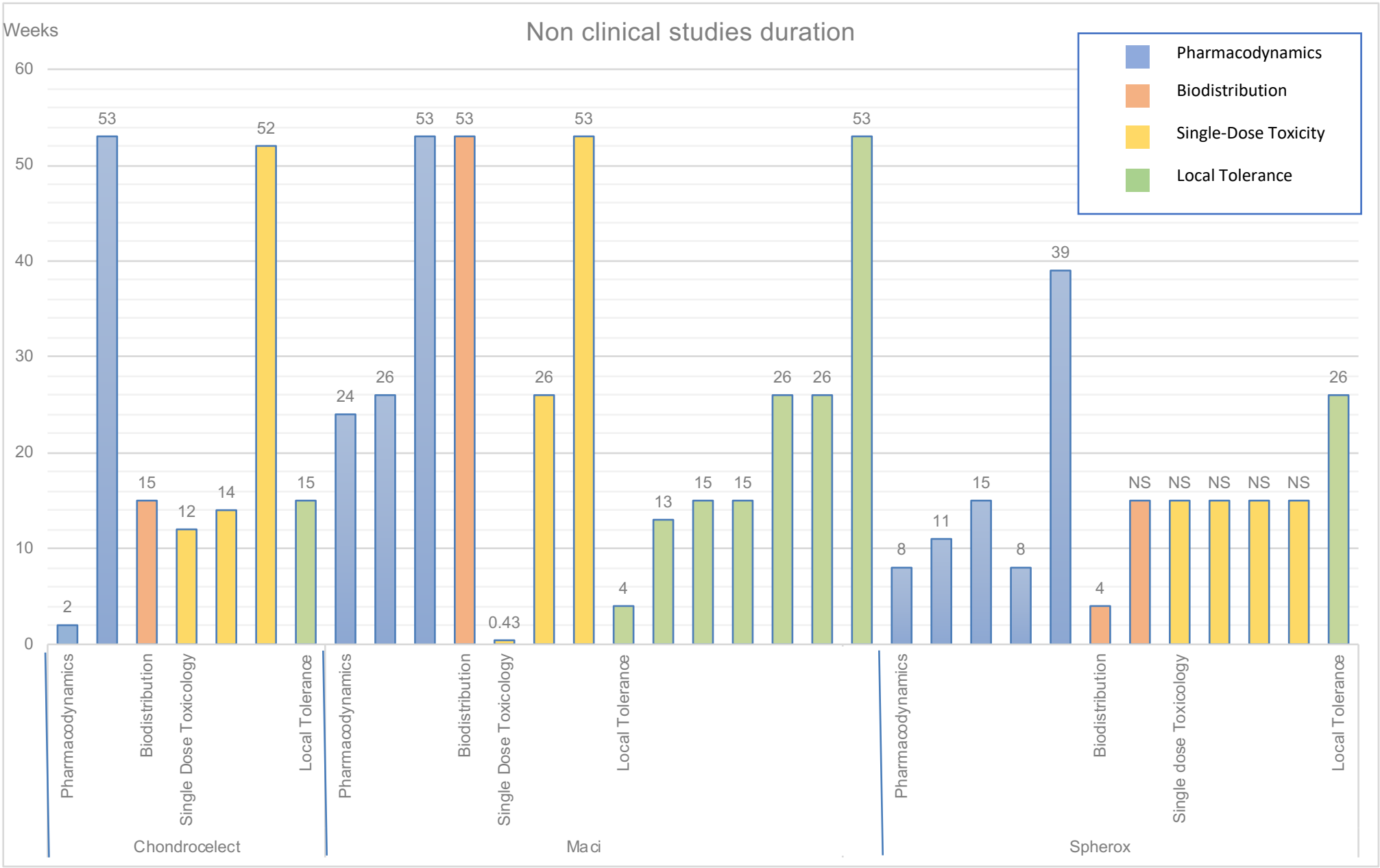


Figure 9 - Duration of non-clinical studies conducted for chondrocyte-based medicinal products

Besides the comparison performed between different ATMPs' EPARs, the scientific document *Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee*[75] will also be considered, since all products under study must follow guidelines available on this document, due to their chondrocyte-based characteristics, in line with named reflection paper.

In the following sections a detailed analysis between different medicinal products' EPARs is presented, as well as conclusions obtained from the previously presented figures and tables, namely figure 8, table 11 and figure 9.

5.1.1. Pharmacology

Primary Pharmacodynamics

When it comes to primary pharmacodynamics, aiming to study the outcomes of medicinal product administration which are related to the desired therapeutics effect, all EPAR documents showed some similarities with regards to this study category. From previously performed analysis it was clear that cell-based products must always include proof-of-principle studies in their development processes and also demonstrate some evidence to proposed mode of action. This was performed in all three ATMPs analysed, and some differences and similarities were encountered in analysed study designs.

Particularly, when considering ChrondroCelect evaluation, proof-of-concept studies started with an ectopic model, further corroborated with an orthotopic model, the goat and with a longer study duration, allowing to assess long-term effects from medicinal product administration. When it comes to MACI, a similar strategy was conducted with first efficacy studies being performed using a smaller animal model, the rabbit and later the horse was used as an orthotopic model to better mimic the outcomes obtained with expected human results. Moreover, Spherox also followed similar study design for efficacy and proof-of-concept, apart from the conduction of *in vitro* studies, discussed afterwards. Primary pharmacodynamics were evaluated in an ectopic model, the mice, during a shorter time period. These results were corroborated by an evaluation in an orthotopic model, the minipig. Finally, a long-term study in sheep was not successful, with no meaningful results acquired, possibly due to an early loss of implanted spheroids leading to an absence of repair tissue.

Furthermore, scientific guideline related to cell therapies supported the idea of both *in vivo* and *in vitro* studies to assess primary pharmacodynamics, with the *in vitro* studies allowing to gather information on tissue morphology and interaction with other tissues. However, only for Spherox *in vitro* studies were conducted, to evaluate spheroids formation and their properties, as well as adhesion and integration that these 3D structures can undergo when in contact with host tissue, a procedure in line with the *Guideline on human-cell based medicinal products* [12] and *Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee* [75].

Apart from this difference encountered, all ATMPs were evaluated when it comes to the repair tissue formation capacity, *de novo* tissue properties, medicinal product integration in the host and degree of

defect filling. Overall these features analysed allowed to assess proof-of-principle, the regeneration of the defect site and formation of new and adequate tissue. Furthermore, it is important to stand out that considering the goal of repairing cartilage defects, common to all compared ATMPs, two assessment reports indicated the performance of biomechanical tests, namely MACI and Spherox. This is an important evaluation to be conducted since the tissue to be formed is exposed to load bearing and therefore must show some particular biomechanical properties, being in line with recommendations present in the *Reflection paper on in vitro cultured chondrocyte containing products for cartilage repair of the knee* [75].

When it comes to the number of primary PD studies performed, no significant differences were encountered and only Spherox warrants some attention, due to conduction of both *in vivo* and *in vitro* tests.

With regard to the duration of the studies performed it is important to stand out that ChrondroCelect only referred the duration of one study, namely 53 weeks in the orthotopic model. Spherox developed a pilot study in minipigs for 8 weeks, considered a small time period to assess all important outcomes, and a pivotal study with longer duration (39 weeks) in sheep. Finally, MACI performed both the pilot and the pivotal study in horses with 26 weeks and 53 weeks of duration, respectively. A longer study allows to evaluate long-term performance of the medicinal products, an important evaluation for the successful administration of every ATMP, intended to be only administered once.

Besides, it is appropriate to conclude on the animal models used. All ATMPs started with studies in small animals but afterwards conducted other studies in larger models. This is relevant since only large models allows to mimic as much as possible the intended mode of administration and the outcomes expected in humans, recommended in the scientific guidelines analysed. Thus, only models such as sheep, goat or horse allow to perform studies in a clinically relevant model, although some primary results can be firstly obtained in smaller and particular ectopic ones.

Secondary Pharmacodynamics

Secondary pharmacodynamic studies, evaluating consequences that are not correlated to the intended therapeutic outcome, were only performed for MACI. These studies were performed *in vitro* and were intended to study cell-cell interactions and cell-matrix interactions, due to the combination of chondrocytes with ACI-Maix membrane in this medicinal product. Furthermore, it is relevant to appreciate that cell properties and interactions' study, for Spherox, were included in primary pharmacodynamic studies and for MACI were conducted in the category under discussion.

For the studied cell-based products only cell and tissue properties, such as phenotype or viability, are important to evaluate since these products are intended for local administration and are expected to show only local outcomes. Thus, if these ATMPs' characteristics are analysed in other performed studies, no dedicated secondary pharmacodynamic analyses are mandatory.

Safety Pharmacology

As well as it occurred with secondary pharmacodynamic, only for MACI safety pharmacology was studied. However, no dedicated studies were performed, and this category was evaluated during proof-of-concept pivotal equine study. This can be justified due to the use of a non-cellular product for this TEP, the ACI-Maix membrane, which may lead to some undesired outcomes in major physiological systems. Since the other two medicinal products are not expected to show effects on unintended locations and are not expected to release any active substance, the absence of safety pharmacology studies is justified.

5.1.2. Pharmacokinetics

With regard to pharmacokinetic studies, one important point stands out, the absence of standard ADME studies for all considered cases, since all these products are cell-based and therefore are not expected to be evaluated with regard to absorption, metabolism and excretion, and it is only relevant to assess medicinal product's biodistribution, as already stated during scientific guidelines comparison within pharmacokinetics' topic.

Thus, for all three chondrocyte-based medicinal products, the biodistribution was evaluated, particularly assessing cell migration capacity from implantation site. Since these products are intended to show local outcomes, their migration capacity must be reduced. Nevertheless, for MACI and Spherox the distribution of cells to most common migration places, namely lymph nodes, lungs, liver and spleen, was assessed, evaluating systemic product dissemination, and thus confirming the absence of migration issues.

When it comes to the number of studies performed and the duration of them, no significant differences were encountered, with the three medicinal products showing evaluation of similar pharmacokinetic aspects. With regard to studies duration, ChrondroCelect and MACI showed adequate durations to evaluate distribution of these medicinal products and Spherox does not included such information on its assessment report.

5.1.3. Toxicology

Single Dose Toxicity

When it comes to single-dose studies, all medicinal products performed dedicated evaluations. This is in line with scientific recommendations previously analysed, considering the expected long life-span that ATMPs must show, only requiring a single administration. More importantly as discussed in section *4.3.1 Single-Dose studies*, for cell-based medicinal products, due to their long-term effects, these studies must be performed with adequate post-administration evaluation periods to assess later outcomes.

When it comes to ChrondroCelect studies, ectopic and orthotopic models were used to perform toxicity studies. When it comes to the ectopic model, a shorter study was conducted in nude mice to assess outcomes of injection of different cell types, such as goat, sheep and human chondrocytes. Furthermore, longer studies were performed with sheep (14 weeks) and goats (52 weeks), evaluating toxicity biomarkers, such as ectopic cartilage formation and inflammation. These toxicity outcomes were evaluated through histological, macroscopic and biochemical studies for tissues and synovial fluid. Moreover, it is relevant to appreciate that ChrondroCelect developed dedicated studies, with adequate post-administration evaluation periods to assess long-term toxicity effects.

With regards to MACI, toxicity studies were also performed in different species, such as one ectopic model, the mice and one orthotopic, the horse, this one included in pharmacodynamic evaluations. Ectopic studies were of shorter duration with evaluation of ACI-Maix membrane toxicity outcomes. On the other hand, studies in horses allowed to evaluate synovial fluid, as studied for ChrondroCelect, assessing inflammation evidences and performing chemistry and histological evaluations.

Spherox's single-dose toxicity studies were performed *in vitro*, only assessing cell senescence and proliferation. *In vivo* studies were part of proof-of concept analyses, an adequate decision since only a single administration is used in these pharmacodynamic studies, avoiding the performance of a separated study with more animal models being used. This product does not justify further dedicated toxicity studies, considering its autologous nature and not showing any membrane attached to it nor including any growth factors or external stimuli during its manufacturing process, as occurs with MACI and ChrondroCelect, respectively. Furthermore, it is relevant to stand out that cells are expanded in culture media that only includes patient's own serum with growth auxiliary purpose, thus not posing any issue when it comes to toxicity and justifying few studies conducted for this category.

Spherox included all of its toxicity evaluations in proof-of concept studies, as discussed before, and the same occurred to two of the three studies for MACI, thus both being in line with recommendations available in *Reflection paper on in-vitro cultured chondrocyte containing products for cartilage repair of the knee* [75]. This difference encountered in the non-clinical study designs when compared to ChondroCelect, with inclusion of safety end points to evaluate toxicity in proof-of-concept studies, for the more recently developed ATMPs, reflect the knowledge and the advice already present in the reflection paper previously named and considered by MACI and Spherox's applicants when creating the developing programs. Since this document still did not existed when ChrondroCelect was developed, no inclusion of safety endpoint was performed in pharmacodynamic studies and dedicated single-dose toxicity studies were conducted, with the major disadvantage of a larger number of animal models being used.

Repeated Dose Toxicity

Repeated dose toxicity studies were not performed for any of the considered tissue engineered products. These decisions can be justified based on products' mode of action and intended single administration, as well as expected long lasting effects, for all of them. Besides, as durations are

considered relevant to assess long-term effects, no need for repeated dose studies was considered, being in line with ATMPs topic discussed in section *4.3.2 Repeated dose studies*.

5.1.4. Genotoxicity

Genotoxicity studies were not performed for all three pharmaceuticals since none of them are expected to interact with genetic material or DNA. Only for MACI, due to the presence of ACI-Maix membrane, it was considered relevant to assess mutagenic potential of matrix components. These approaches were in line with the previously studied *Guideline on human-cell based medicinal products* and the results obtained during chapter 4 study.

5.1.5. Tumorigenicity

Tumorigenicity studies are of major relevance for cell-based medicinal products, due to their migration and proliferation capacity, which lead to relevant tumorigenic potential.

The ChrondroCelect and Spherox's non-clinical data package include tumorigenicity studies. ChrondroCelect evaluation was in line with scientific recommendations, conducting *in vitro* studies to assess cell properties, such as senescence after some passages, thus studying product potential to induce neoplastic alterations. When it comes to Spherox, these studies were performed in mice, with the evaluation of tumor formation and migration from site of administration. This procedure is in line with the scientific suggestion to conduct studies on migration and proliferation capacity. Moreover, these *in vivo* assessments were performed for 26 weeks, considered the adequate minimal duration to evaluate tumorigenic potential, being incorporated in the same study that evaluates pharmacodynamics and local tolerance.

With regard to MACI, no dedicated long-term tumorigenicity studies were conducted. This approach was in line with the negative results obtained for the mutagenic potential of the ACI-Maix matrix, during previously mentioned studies, included in genotoxicity section. When it comes to the cellular material present in this medicinal product, human chondrocytes expanded for autologous transplantation were studied for chromosomal stability, with results indicating a stable cellular karyotype for long periods. Both of these outcomes justified the absence of long-term tumorigenicity evaluations.

5.1.6. Reproduction Toxicity

Reproduction toxicity was not evaluated for any of the three medicinal products considered. These decisions can be justified due to products' cellular nature, local administration and low migration capacity, consequently not expected to pose any risk for fertility or pre and post-natal development. Thus, these properties denote low risk for reproduction and developmental toxicity issues.

5.1.7. Local Tolerance

Local tolerance is one of the categories that had major relevance for these medicinal products' non-clinical development processes, being performed for all ATMPs considered within chondrocyte-based category, with a particular relevance for MACI. Some relevant dissimilarities were found for local tolerance studies between the three medicinal products considered, due to their composition.

When it comes to ChrondroCelect, local tolerance evaluation was conducted during toxicological studies in orthotopic models, allowing to evaluate any inflammatory outcomes that might occur due to medicinal product administration and reducing the number of dedicated studies performed and the also the animal models used.

For MACI, several local tolerance studies were performed, some incorporated in toxicity and proof-of-concept studies and other dedicated to this non-clinical category. The major issue when it comes to this product is the ACI-Maix membrane, that might lead to some inflammatory response and for that reason, most of these studies were conducted to assess local tolerance to this component. Different animal models were used, such as rats, during different time points, particularly 4, 13 and 26 weeks, with the last time-point already allowing to evaluate local tolerance persistence, as well as membrane degradation. To study this toxicity category, hemorrhage, necrosis and foreign body response were evaluated. When considering the orthotopic model, the synovial tissue was studied to assess some alterations that might occur due to inflammatory issues.

Spherox's local tolerance study were performed for 26 weeks in sheep, the same study that also evaluated pharmacodynamics and single-dose toxicity, assessing the inflammatory outcomes that might occur due to spheroids implantation. Since this product does not include any component of higher toxicity hazard and has an autologous nature, it is expected to be well tolerated, thus not showing any adverse effects.

When comparing the number of studies performed and the duration of them for the three medicinal products analysed, it was clear that MACI showed the larger number of local tolerance studies, with some included in proof-of-concept studies. This is acceptable due to the presence of an external matrix in this medicinal product, leading to the necessity of evaluating its outcomes and possible local adverse effects. With regard to the duration of the studies performed, information was available for MACI and Spherox, with appropriate post-administration time points, which allowed detection of adverse effects in the local of administration.

5.2. Classification of ATMPs according to their characteristics and risks

After the analysis performed with ATMPs' assessment reports, some differences and similarities were encountered, besides the ones previously analysed, for chondrocyte-based medicinal products. From the evaluation of ATMPs' mode of action, intended therapeutic effect and non-clinical studies' designs, it was considered relevant to develop a new classification system, particularly based in medicinal products' mode of administration and risks that each ATMP might pose.

Firstly, to obtain the new classification system, medicinal products were compared according to their application site, which allowed to comprehend that some use a local administration and others a systemic one, such as an intravenous delivery. Moreover, it was also possible to understand that some ATMPs under study showed local risks and other presented systemic ones. Thus, the classification present afterwards was developed and each ATMP analysed was included in a single category. In the following sections it is also provided a discussion on the reasons that support the classification performed for each ATMP.

Furthermore, the assessment reports' analysis allowed to gather some evidence of risk-based approach's application on different non-clinical development studies. From the knowledge acquired during ATMPs' development processes, in particularly when it comes to their mode of action, way of administration and likely outcomes, it is possible to identify potential risks and risk factors related to medicinal products' use. This awareness is crucial when developing a non-clinical program, since it allows to conduct a meaningful non-clinical analysis, determining the extent and type of evaluations to be performed. This approach is an on-going process that allows a flexible design of non-clinical studies and consequent tailoring of such development steps, bearing in mind the already known ATMPs' features and considering the information acquired in previously conducted studies, since beginning of non-clinical evaluation. All of this data gathered together influence each step of the analysis performed, as more and more information is obtained [38]. Thus, influence and consequences of the risk-based approach implementation for non-clinical development processes were identified, during assessment reports' appraisal and must be considered when discussing the evaluation program conducted.

Therefore, discussion of each ATMP regarding the newly created classification was conducted in line with the risk-based approach and some conclusions were obtained when it comes to the way this methodology influences the non-clinical studies performed.

5.2.1. ATMPs with a local application and posing local risks

Belonging to the first category, it was possible to classify chondrocyte-based medicinal products, namely Spherox, MACI and ChrondroCelect, as ATMPs delivered through a local administration site and posing local risks.

Firstly, two of the chondrocyte-based medicinal products are presented as a suspension for local implantation, particularly ChrondroCelect and Spherox. On the other hand, MACI is delivered as a collagen membrane where cells are seeded. Besides from their local administration sites, considering that these ATMPs are not expected to contact blood circulation, they are not expected to pose any risks away from implantation site, thus only posing local risks inherent to their use.

These two key properties of analysed chondrocyte-based medicinal products, tailor the non-clinical studies performed, considering a risk-based approach for some cases. Before further discussion when it comes to the way risk-based methodology influenced non-clinical development programs, it is important to stand out that key differences were encountered between development programs of chondrocyte-based medicinal products approved during different time points.

Particularly, ChrondroCelect was authorized for market entrance in 2009 and only after that, in 2013, a practical guideline from CAT was made available, to support risk-based approach implementation, the *Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products* [38]. On the other hand, Spherox received the European marketing authorization in 2015, already after named guideline was made available. This allowed an easier implementation of this methodology, during Spherox's development process, following practical advice present in the available scientific guideline. One clear evidence of this methodology implementation for this ATMP is the reference to risks and risk factors as recommended in previously named guideline, a feature only encountered for Spherox, when compared to the other two chondrocyte-based medicinal products. Also, MACI came to the market soon after *Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products* [38] was released, thus differences in the way non-clinical studies were conducted are possible to find when compared to ChrondroCelect, but there is an absence of so clear evidence of this methodology application as it was found during Spherox's evaluation. Thus, it was understood that the existence or absence of practical guidance for risk-based approach implementation influences the way non-clinical studies are conducted, with ChrondroCelect not showing as great evidence of this approach implementation as MACI, and particularly Spherox, do.

Further discussion on specific evidence of this methodology application is presented next. Besides, this section is divided according to the risks considered for chondrocyte-based medicinal products and a brief discussion on how each risk was evaluated for the three different ATMPs will be provided for each risk considered.

Lack of efficacy

All medicinal products pose the risk of lack of efficacy, but since chondrocyte-based medicinal products are intended to restore knee's functional properties, efficacy is an important feature to be evaluated for these products, with specific functional studies being performed, such as testing of biomechanical properties like aggregate modulus. These studies were performed, as part of primary pharmacodynamics, for MACI and Spherox and only for ChrondroCelect such functional evaluation was not conducted. Besides these biomechanical properties evaluated for the MACI and Spherox, all ATMPs underwent structural properties evaluation of *de novo* tissue formation, allowing to prevent lack of efficacy issues. These structural issues are mainly related to delamination, which leads to lack of efficacy in restoring asymptomatic knee's biomechanical properties and ultimately possible graft failure.

Graft Hypertrophy

Besides lack of efficacy, chondrocyte-based medicinal products may pose hypertrophy issues, due to inappropriate cell proliferation. This hazard leads to adverse events, such as pain or joint swelling, not allowing to restore normal knee's functional properties, without symptoms, as desired.

This risk was assessed with evaluation of tumor development, analyzing cell senescence and ectopic tissue formation, through biodistribution and tumorigenesis studies performed.

Local adverse effects – Local Inflammation

Since chondrocyte-based medicinal products are intended to show local therapeutic effects and are provided through a local implantation site, *in situ* adverse events are important risks to be assessed, since early development phases, such as non-clinical evaluation. Due to this risk relevance, all three ATMPs considered for this category underwent local tolerance studies, some being part of other toxicological studies, such as single-dose evaluations, that as a whole allowed to gather relevant data related to medicinal products' toxicity. Studies performed included assessment of Graft versus Host Disease, inflammatory signs and tissue necrosis. They were conducted in different animal models and with different time points, allowing to assess earlier and later negative outcomes.

Furthermore, it is important to highlight the number of studies performed for MACI, higher than the number of studies performed for the other two ATMPs within this category. This can be justified due to the presence of a collagen membrane that may lead to adverse effects in administration site and negative outcomes due to its degradation process. Thus, all of these external matrix's risks must be assessed during local tolerance studies, following a risk-based methodology.

Conclusively, studies assessing each key risk that these medicinal products poses are important to identify, as soon as possible in the development process, the main safety and efficacy issues that these ATMPs pose, particularly in local administration site. Following a risk-based approach, particularly for MACI and Spherox, it was possible to conduct only relevant studies considering medicinal product characteristics, reducing the number of studies performed and the number of animal

models used. Furthermore, it allowed to focus the evaluation program in important assessments, acquiring relevant non-clinical data. One particular example of risk-based approach for these medicinal products is the absence of genotoxicity studies, as well as repeated dose toxicity studies, being justified, respectively, due to cell nature of chondrocyte-based medicinal products and not expected influence in general reproductive function and fertility, as well a single administration procedure. Thus, these evaluations are not relevant to be conducted and are omitted from chondrocyte-based medicinal products' non-clinical evaluations.

5.2.2. ATMPs with a local application and posing systemic risks

Belonging to the category of ATMPs with a local administration approach and posing systemic risks is Alofisel. This medicinal product is administered through a local intralesional injection, as a suspension, particularly in the anal fistula wall. On the other hand, due to its migration capacity and possible blood circulation entrance risks inherent to its use might be systemic.

Before further discussion on Alofisel specific risks and how data related to identified risks were obtained, it is important to underline that Alofisel was approved for market entrance in 2017, after *Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products* [38] was released. Thus, it was possible to identify clear evidence of this approach implementation throughout Alofisel assessment report, allowing to minimize the number of studies performed and optimizing the relevant data acquired. The main risks related to this ATMP use are depicted and discussed next.

Tumorigenicity

Alofisel is a cell therapy, containing expanded adipose stem cells from allogenic source, intended to have an immunomodulatory outcome. However, due to stem cell proliferation capacity, it is relevant to assess cell proliferation, senescence and telomerase activity. These cells' characteristics allow to comprehend to which extent there is a risk of tumor formation. From studies conducted, both *in vitro* and *in vivo*, it was possible to determine that no risk of tumorigenic potential was identified for Alofisel. Nevertheless, it is important to highlight the possible remaining differentiation capacity of expanded adipose stem cells, that as the risk for tumor formation, constitute a relevant issue to be addressed later in clinical studies. Non-clinical evaluation allows to determine a low undesired differentiation capacity but does not allow to acquire knowledge on how expanded adipose stem cells will behave in humans.

Migration

Although Alofisel is administered locally, cells may show some migration capacity from injection site, due to their stem cell nature. To evaluate possible distribution to non-target sites pharmacokinetic studies must be performed, allowing to evaluate unwanted outcomes, such as possible ectopic tissue formation.

During Alofisel biodistribution assessment, it was considered relevant to use intended clinical route, namely intrarectal and perianal, evaluating migration from local administration site, and an intravenous route to evaluate systemic medicinal product distribution. Since in Alofisel administration site there is a high number of capillaries and blood vessels, it is possible that the medicinal product enters the systemic circulation inadvertently, possible leading to adverse events, such as ectopic tissue formation. Thus, to evaluate distribution and engraftment when this unfavorable event occurs, it was considered relevant to perform biodistribution evaluation using an intravenous route. Furthermore, it is relevant to stand out that possible Alofisel administration into blood vessels leads to the necessity of performing safety pharmacology studies, according to risk-based approach, evaluating outcomes in major physiological systems that this medicinal product might reach.

Immunotoxicity

Considering Alofisel immunoregulatory expected effects, some adverse events might occur, due to unwanted immune response. This medicinal product is intended to reduce inflammatory signs, allowing tissues around the fistula to heal, thus regulating immune response through interaction with different lymphocytes and cytokines. This mechanism of action made it relevant to assess Alofisel interaction with NK cells, T cells, T_{reg} cells and cytokines during immunogenicity studies, to evaluate possible adverse events before first-in-human studies.

Furthermore, since cells present in Alofisel come from an allogenic source, a possible alloimmunoreaction may occur, with the recipient developing an immune response to allogenic stem cells. This adverse event may lead to unwanted Alofisel outcomes, or even loss of intended function, without healing of anal fistula.

5.2.3. ATMPs with a systemic application and posing local risks

Heparesc was classified as an ATMP delivered systemically and posing local risks. This medicinal product is administered using portal vein bloodstream, as a concentrated cell suspension, posing relevant risks due to its administration approach. Thus, most relevant hazard associated with Heparesc's use is discussed next.

Local Risks due to administration procedure

Considering Heparesc composition, intended mode of action and administration approach, it was clear to recognize that key unfavorable aspects regarding to its use are related to the administration approach, an intravenous delivery, particularly an intraportal cannulation.

Previously documented studies concluded that injection of cell suspensions through small catheters via portal vein bloodstream lead to possible complications such as portal vein thrombosis and pulmonary embolism. These complications occur due to obstruction of sinusoidal spaces, which afterwards leads to cells' distribution to the lungs [76]. Such hazards might conduct to undesirable outcomes and lack of efficacy from medicinal product use.

Thus, to assess such threats, during non-clinical development studies, it was relevant to evaluate parameters allowing to assess efficacy of administration procedure. Firstly, during biodistribution studies, sinusoidal uptake capacity was evaluated, as well as hemodynamic alterations. Furthermore, also systemic distribution to non-target places was assessed, with identification of the lungs as the organ to where infused hepatocytes might distribute. Therefore, evaluation of pulmonary shunting was also performed, as well as cells elimination from this organ. No further tissues were evaluated, bearing in mind the risk-based approach, as distribution to other non-target sites are not expected.

When it comes to toxicity studies performed, during repeated dose evaluations, administration approach was studied, with assessment of clinical signs such as body temperature, heart rate modifications, occurrence of fibrosis, signs of hyperventilation and portal hypertension.

5.2.4. ATMPs with a systemic application and posing systemic risks

When it comes to ATMPs delivered using a systemic administration and showing systemic hazards, Zalmoxis was the ATMP classified as the medicinal product belonging to this category. This cell-based therapeutic product poses some risks due to its particular characteristics, namely the genetic modification of T cells administered to the patient. Risks associated to Zalmoxis use are presented and discussed next.

Graft versus Host Disease

Although it is relevant to study ATMPs' mechanism of action for all medicinal products considered during proof-of-concept studies, particular attention must be paid to Zalmoxis functional assessment. Such evaluation of ATMP's mode of action is of major relevance, since in case the treatment fails immunotoxicity events may occur, leading to a Graft versus Host Disease scenario.

This ATMP is delivered intravenously, as an infusion of T cells genetically modified to contain a suicide gene which respond to ganciclovir. Administration of this drug allows to selectively kill T cells containing the suicide gene, when Graft versus Host Disease occurs, and proliferation of such cells is verified. Considering this mechanism of action, it is necessary to perform functional studies when it comes to Zalmoxis reaction upon ganciclovir administration, assessing T cell destruction when GvHD occurs. If response to ganciclovir is not as expected, T cells will continue to proliferate, which can lead to serious adverse events and consequently conduct to undesired administration of immunosuppressive drugs. Thus, functionality studies were performed during primary pharmacodynamic evaluations. These studies allowed to assess which was the response of T cells containing the suicide gene to ganciclovir and in this way assessing efficacy of suicide system used in Zalmoxis.

Furthermore, since this ATMP intends to help restore immune system, during quality aspects evaluation, expression patterns of T-cells co-receptors were assessed, as well as activation markers. These data served as basis for *in vitro* non-clinical pharmacology studies.

Carcinogenicity

Zalmoxis use might pose carcinogenicity issues due to possible oncogenic risk associated with this medicinal product use.

This hazard is mainly related to the potential of insertional mutagenesis due to vectors integration in the human genome. Insertion of such vectors occur in specific locations, named Common Insertional Sites (CIS), next to proto-oncogenes which may lead to their activation, promoting oncogenic potential [77]. To assess such potential hazards, insertional patterns, gene expression profile and clonality of cell populations were studied during *in vitro* carcinogenicity evaluation. Further *in vivo* studies were performed by different groups of investigators, evaluating oncogenic potential of transduced cells containing genes inserted using a retroviral vector. These studies allowed to assess low oncogenic risk posed by Zalmoxis administration, allowing to gather data concerning potential oncogenic issues, although they are not considered traditional carcinogenicity studies.

5.3. Overview of shared non-clinical testing properties for considered ATMPs

From the extended research performed to find meaningful regulatory documents, bearing in mind the scope of this thesis, it was not possible to find a guideline providing recommendations for non-clinical studies, required for FIH evaluation when considering ATMPs, since such regulatory document is still not available due to ATMPs relative novelty. However, the document *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16] was considered relevant to be analysed in comparison with overall conclusions gathered throughout previously discussed study phases. Though recommendations present in this guideline are not within the scope of advanced therapy medicinal products, general considerations may be applied to ATMPs' development processes [50]. Nevertheless, ATMPs include a broad range of medicinal products, with very diverse features such as mode of action or intended therapeutic effects, but general aspects were found to be common within considered advanced therapy medicinal products, allowing to assess general non-clinical requirements essential for further initial administrations in humans.

The previously referred guideline on FIH studies include recommendations for non-clinical studies, as well as quality issues, these last ones not within the scope of this thesis. Non-clinical considerations, available in this guideline, include efficacy and safety concerns, particularly pharmacodynamics, pharmacokinetics and toxicology, which were found to be in common with all evaluated ATMPs' non-clinical development processes. Thus, it was considered relevant to discuss such studies and compare them with the recommendations available in the referred guideline. To allow a clearer discussion further evaluation was divided in different sections.

5.3.1. Overview of animal models used during non-clinical development programs

Firstly, one important point to stand out, amended in the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16] and throughout all assessment reports evaluated, is the relevance of the animal models used during non-clinical studies. Such models should allow to obtain significant outcomes, mimicking the intended therapeutic effects and possible adverse events and allowing to reflect human disease in all its characteristics. Thus, both small models, for example mice, and larger animal models, such as sheep or horses, must be considered to reflect as much as possible human expected outcomes [78].

One important example of the relevance of an adequate animal model's choice is obtained when looking at the animal used to evaluate chondrocyte-based medicinal products. For such ATMPs, namely ChrondroCelect, MACI and Spherox, studies started to be performed in smaller animal models, allowing to obtain first proof-of-concept results. Further functional studies, crucial to predict success of the intended therapeutic outcomes, were developed using larger animal models particularly horse, goat or sheep. Such models proved to be relevant due to higher similarities of anatomical and physiological characteristics, allowing to acquire relevant outcomes when conducting biomechanical studies [79], essential for the success of chondrocyte-based medicinal products, as discussed previously in section 5.1.1.

On the other hand, for the remaining evaluated ATMPs, namely Alofisel, Zalmoxis and Heparesc, no preclinical studies were performed in larger animal models, since the expected therapeutic effects of these ATMPs do not depended on functional outcomes and anatomical features, as much as chondrocyte-based medicinal products do. For Alofisel and Zalmoxis, medicinal products of allogeneic origin, immunocompromised small animal models (i.e. mice and rats) were used allowing to evaluate possible therapeutic outcomes or adverse events and avoiding immediate immune recognition of human cells and their consequent rejection, due to xenogeneic medicinal product origin.

When it comes to Heparesc, a rabbit animal model and rabbit liver cells were used to assess possible therapeutic outcomes and adverse events. For this ATMP, it is important to stand out that despite the existence of a more relevant animal model mimicking one of the intended indications, rabbits and rabbit cells were used in an attempt to correlate non-clinical outcomes obtained with further clinical results. Thus, it was possible to generally support the concept of intraportal hepatocytes perfusion and indirectly evaluate proof-of-concept outcomes, although this animal model of disease lacks demonstration of human expected outcomes when it comes to efficacy of the ATMP [67].
Further, it is also relevant to bear in mind the possible weakness of studies, with animal model's cells as observed with Heparesc, stated in the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16], where it is identified the reduced value of *in vivo* studies performed with cells from animal models when there may exist some species-specific therapeutic outcomes and when it is not possible to evaluate an animal model with the exact same disorder as the therapeutic intended target.

Additionally, recommendations related to the use of *in vitro* models can be found in the considered guideline for FIH studies as well as in all considered ATMPs' non-clinical development processes, apart from Heparesc, for which only animal studies were performed. Such *in vitro* systems are crucial, particularly allowing to study human cell structures and avoid misinterpretations of possible human use consequences caused by animal models' outcomes. Furthermore, as it was possible to see throughout all ATMPs' non-clinical development processes, integration of different information sources, such as *ex vivo*, *in vivo* and *in vitro* studies, allows to perform a more accurate extrapolation to human expected outcomes, as also advised in the previously mentioned guideline.

5.3.2. Overview of pharmacodynamic studies performed

Furthermore, the mentioned guideline addresses primary pharmacodynamic studies as intending to provide outcomes on product's mode of action and functional outcomes, a recommendation followed in every considered ATMP's non-clinical program evaluated.

Besides, secondary PD studies must be considered whenever some not intended outcome might exist and must be performed according to the risk-based approach, as occurred with MACI, where interactions between collagen membrane and cells were evaluated to assess successfulness of the medicinal product.

Both primary and secondary pharmacodynamic studies are recommended to be performed in the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16] using both animal models and *in vitro* studies, a recommendation followed for Zalmoxis, MACI and Spherox.

Additionally, safety studies were considered for the evaluated ATMPs on a case-by-case basis. Thus, if the ATMP is expected to interact with major physiological systems, particularly cardiovascular, respiratory, renal and central nervous ones, safety evaluations must be performed as occurred with Alofisel, an ATMP with a local administration procedure but which might pose some systemic hazards.

5.3.3. Overview of pharmacokinetic studies performed

When it comes to pharmacokinetic studies, as amended in the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16], all ATMPs performed pharmacokinetic evaluations that allowed to support outcomes obtained in pharmacodynamic studies and further assess biodistribution.

Firstly, it is important to stand out that for all ATMPs, standard pharmacokinetic studies (i.e. ADME) were not considered relevant, due to medicinal products cell-based nature and only biodistribution was considered appropriate to be assessed. For chondrocyte-based medicinal products, larger animal models were used to study biodistribution from implantation site. When it comes to Alofisel also

biodistribution evaluation was performed and allowed to conclude on possible migration to major physiological systems and consequently support the conduction of safety pharmacology studies. For Zalmoxis, biodistribution studies were also conducted although they were not of major relevance according to the risk-based approach and expected possible hazards of this ATMP administration. Finally, when it comes to Heparesc, pharmacokinetic studies allowed not only to evaluate if cells would be retained in the liver or could possibly enter systemic circulation, but also to study local outcomes of cells administration assessing portal hemodynamic alterations and sinusoidal uptake capacity.

5.3.4. Overview of toxicology studies performed

Finally, as well as it occurred with pharmacodynamic and pharmacokinetic studies, also toxicology evaluation was conducted during non-clinical studies of all evaluated ATMPs. When it comes to this category, differences were found according to the ATMP under evaluation, following a case-by-case approach, thus assessing the relevance of toxicology studies to be performed for each medicinal product under appraisal. Recommendations present in the *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products* [16] state the importance of performing toxicology evaluations and assessing possible adverse events due to medicinal product's administration. All ATMPs evaluated were in line with this recommendation, conducting toxicology evaluations, whether using dedicated studies or integrating toxicology end points in other evaluations performed, as occurred with Zalmoxis, for which single-dose toxicity was evaluated during pharmacodynamic studies.

When it comes to repeated dose toxicity studies some important points are relevant to discuss. For all the six considered ATMPs, repeated dose toxicity studies were conducted following a case-by-case approach, whenever the intended mode of administration and expected mechanism of action justified such evaluations. For chondrocyte-based medicinal products, namely ChrondroCelect, MACI and Spherox, repeated dose studies were not performed, which was justified due to medicinal products' intended single administration and expected long-term therapeutic outcomes, as stated before in section 5.1.3. On the other hand, repeated dose evaluation was performed during Heparesc non-clinical studies, since this ATMP is intended to be administered using six daily doses, thus justifying the relevance of repeated dose studies.

Furthermore, it is due to long-term expected outcomes for cell-based medicinal products that it is important to perform toxicology studies during long enough time periods to obtain clinically relevant outcomes, a recommendation considered for all ATMPs, with information on studies duration. One example of long-term toxicology evaluation performed is obtained with MACI, for which long single-dose evaluation was conducted for 53 weeks, allowing to acquire clinically significant outcomes.

6.Conclusions

Advanced therapy medicinal products have been exhibiting great impact in healthcare over the past several years, allowing to obtain therapeutic positive outcomes to so far unmet medical needs and life-threatening conditions. However, as time goes by, the number of ATMPs already available in the market remains quite low, when compared to the positive impact this innovative pharmaceutical field has showed. This unpleasant reality is justified with the complex nature of these medicinal products and their patient-specific characteristics, making their market entrance an even more arduous procedure when compared to standard medicinal products.

Due to ATMPs relative novelty and complex nature, there are still an unsatisfactory number of regulatory documents identifying the specific requirements for these medicinal products' market entrance approval. It is also important to stand out the absence of a single scientific guideline when it comes to requirements for first-in-human studies that includes gene therapy, cell-based and tissue engineered products. Such guideline could be analysed in conjunction with Regulation (EC) No 1394/2007 [5] and other regulatory documents concerning ATMPs, allowing broader guidance to be given and helping in non-clinical development process. The comparative analysis performed during this study, with the use of NVivo software, focused on the preclinical stage and requirements for first-in-human studies with regard to cell-based medicinal products, assessing the lack of specific guidelines and recommendations for this important pharmaceutical development phase. Due to the absence of a specific guideline concerning FIH requirements for all ATMPs' categories, it was considered relevant firstly to compare guidelines assessing preclinical data package for different medicinal product categories, namely small molecules, biopharmaceuticals, cell-based medicinal products and gene therapy and secondly to analyse different ATMPs' non-clinical studies.

The first study performed, comparing four guidelines, namely *ICH M3(R2) – Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals* [10], *ICH S6- Preclinical safety evaluation of biotechnology-derived Pharmaceuticals* [11], *Guideline on Human Cell-based Medicinal Products* [12] and the *Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products* [13], allowed to gather some conclusions on preclinical development goals. From the comparative analysis performed for the four named guidelines it was possible to comprehend that the core goal of preclinical studies was identical independently of the medicinal product under evaluation. All non-clinical studies must allow to acquire relevant data on safety and efficacy of the intended mechanism of action and mode of administration, allowing to obtain proof-of-principle information and assess possible human adverse events from extrapolation of results obtained from the animal models used and *in vitro* studies performed. Although these goals are in common for all medicinal products considered, the relevant studies to be performed are dependent on the characteristics of the pharmaceutical under study. Thus, although standard study goals are relevant for all medicinal products, the way preclinical data is acquired and which results are expected are highly dependent on

product characteristics and intended therapeutic effect. The best example of diverse requirements for ATMPs and standard pharmaceutical products are found for pharmacokinetic evaluations. When it comes to small molecules, absorption, distribution, metabolism and excretion must be assessed and on the other hand, for cell-based medicinal products, cells migration and biodistribution must be considered. Thus, although these studies are distinct they allow to obtain information with regard to the same category, pharmacokinetics.

Further analysis performed, and divided in three major phases, intended to compare and evaluate non-clinical studies performed for ATMPs that showed some similar and distinct features, namely ChrondroCelect, MACI, Spherox, Alofisel, Zalmoxis and Heparesc.

Firstly, ChrondroCelect, MACI and Spherox were compared and classified as chondrocyte-based medicinal products, considering the similarities found when it comes to the intended therapeutic effects, mode of action and method of administration. From the analysis performed with these ATMPs' EPARs it was possible to encounter significant similarities when it comes to preclinical studies performed, justified by the likenesses also found between chondrocyte-based medicinal products' characteristics. For instance, for all of them, larger animal models were evaluated to better mimic human expected outcomes and perform relevant functional studies to assess successfulness of the evaluated medicinal product. Another relevant point was the absence of repeated dose studies for chondrocyte-based medicinal products since all intended to be administered once and to show long-term effects. Furthermore, local tolerance evaluation was conducted for all three ATMPs, since all intended to show a local therapeutic outcome and local administration procedure, making local tolerance studies important to assess possible adverse events *in situ*.

The second case studies phase was developed bearing in mind the risk-based approach and more specifically the *Guideline on the risk-based approach according to Annex I , part IV of Directive 2001 / 83 / EC applied to Advanced Therapy Medicinal Products* [38]. This methodology is considered important to evaluate ATMPs, considering their complex nature and case-by-case development process, allowing to assess relevant studies to be performed acording to the risks and risk factors identified for each medicinal product. Thus, the application of the referred approach, in particular to the non-clinical evaluation process, allows to more flexibly tailor the studies to be implemented and accurately assess safety and efficacy, crucial to initiate first administration in humans.

Therefore, ATMPs were classified according to their intended method of administration and possible risks inherent to their use. Thus, for each medicinal product, the main expected risks were identified and analysed and further analysis was conducted on how such hazards were assessed during preclinical studies performed and how risk-based approach influenced preclinical evaluations. The chondrocyte-based medicinal products were classified as ATMPs with local administration and posing local risks, thus the main hazards identified were the lack of efficacy evaluated during proof-of-concept evaluation, graft-hypertrophy, evaluated during tumorigenesis and biodistribution studies assessing unwanted cell proliferation and local inflamation, evaluated during local tolerance analyses. Secondly, Alofisel was classified as an ATMP with local administration and posing systemic risks, for which tumorigenicity was identified as an hazard, assessed using *in vitro* and *in vivo* studies. Besides,

migration was also recognised as a possible risk and evaluated in biodistribution studies, as well as immunotoxicity, evaluated during immunogenicity studies. Heparesc was classified as an ATMP with systemic mode of administration and posing local risks due to its delivery method, via intraportal cannulation. Thus, its mode of administration was assessed, allowing to evaluate its feasibility and possible adverse events, during biodistribution and repeated dose toxicity studies. Finally, Zalmoxis was classified as an ATMP delivered systemically and posing systemic risks. Particularly, Graft versus Host Disease was identified as the main hazard, in case the suicide system is not functional, being studied during primary pharmacodynamic evaluations. From this evaluation it was possible to comprehend that all medicinal products developed after guidance on risk-based approach was made available had a bigger influence of this methodology implementation in their non-clinical development. This conclusion is particularly clear when comparing ChrondroCelect, approved before guidance on the risk-based approach, with MACI and Spherox, approved after guidance on this methodology implementation was made available.

Finally, the third case studies stage intended to gather general insights of non-clinical evaluation programs and conclude on the main requirements for FIH studies. Thus, it was possible to comprehend that the goals of all studies performed were common to all ATMPs assessed, as well as the animal models used, with particular focus on large animal models, used for all chondrocyte-based medicinal products, to perform relevant functional studies and mimic human anatomical and physiological characteristics. Besides it was possible to comprehend that some studies were only performed if the risks identified justified so, particularly local tolerance, repeated dose toxicity studies, carcinogenicity and immunotoxicology. Furthermore, all ATMPs conducted biodistribution studies, as well as pharmacology and toxicology assessments, even if not performed as dedicated studies, as occurred for toxicology evaluation of Zalmoxis, assessed during pharmacodynamic analyses.

Conclusively, the comparative study conducted for this thesis development allowed to gather some conclusions on recommendations and requirements for FIH studies, after the analyses of relevant scientific guidelines and the six different ATMPs' preclinical evaluation processes. Further from the conclusions obtained, one major point stands out from the study conducted, the relevance of a dedicated scientific guideline on preclinical studies required to initiate FIH administrations for ATMPs, containing recommendations according to the risk-based approach and bearing in mind the complex and diverse nature of all medicinal products classified as an advanced therapy medicinal product, including cell-based medicinal products, gene therapies and tissue-engineered products. Although this pharmaceutic field still remains very complex, we expect that during the next several years more regulatory documents and guidelines within the topic will be made available. Unfortunately, ATMPs available in the market are still very few, thus it is crucial to simplify their development and further market entrance, increasing the number of patients using these innovative medicinal products as well as increasing patient therapeutic outcomes to so many unmet medical needs that still exist.

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