Sofia Pereira Carvalho

# Modeling Lysosomal Storage Disorders in an Innovative Way: Establishment and Characterization of Stem Cell Cultures From the Dental Pulp of Mucopolysaccharidoses Patients

Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica orientada pela Doutora Sandra Catarina da Conceição Alves e pelo Professor Doutor Luís Pereira de Almeida e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Fevereiro de 2023

UNIVERSIDADE Ɖ
COIMBRA

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***"This isn't magic, it's Science"***

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## List of Abbreviations

**ARSB** Arylsulfatase B

**BBB** Blood Brain Barrier

**BGAL** β-galactosidase

**BMMSC** Bone Marrow Mesenchymal Stem Cells

**C6S** Condroitin-6 sulfate

**CNS** Central Nervous System

**CRISPR** Clustered regularly interspaced short palindromic repeats

**CS** Chondroitin Sulfate

**DFSCs** Dental Follicle Stem Cells

**DMSCs** Dental Mesenchymal Stem Cells

**DPSCs** Dental Pulp Stem Cells

**DS** Dermatan Sulfate

**DT** Alpha-Tocopherol

**ER** Endoplasmic Reticulum

**ERT** Enzyme Replacement Therapy

**GAGs** Glycosaminoglycans

**GALNS** N-acetylgalactosamine 6-sulfatase

**GFAP** Glial Fibrillary Acidic Protein

**GNS** N-acetyl-glucosamine-6-sulfatase

**GUSB** β-glucuronidase

**HGSNAT** Heparan-alpha-glucosaminide N-acetyltransferase

**HPBCD** Hydroxypropyl--cyclodextrin

**HS** Heparan Sulfate

**HSC** Hematopoietic Stem Cells

**HSCT** Hematopoietic Stem Cell Transplantation

| | |
|---|---|
| **HYAL1** | Hyaluronidase 1 |
| **IDS** | Iduronate-2-sulfatase |
| **IDUA** | α-L-Iduronidase |
| **iPSCs** | Induced Pluripotent Stem Cells |
| **KS** | Keratan Sulfate |
| **LAMP** | Lysosomal associated membrane protein |
| **LSDs** | Lysosomal Storage Diseases |
| **M6P** | Mannose-6-Phosphate |
| **MPS** | Mucopolysaccharidosis |
| **MSCs** | Mesenchymal Stem Cells |
| **NAGLU** | α -N-acetylglucosaminidase |
| **NCL** | Neuronal Ceroid Lipofuscinosis |
| **NMD** | Nonsense-mediated mRNA decay |
| **NPC** | Neural Progenitor Cells |
| **NSC** | Neural Stem Cells |
| **PDLSCs** | Periodontal Ligament Stem Cells |
| **PTC** | Premature Termination Codon |
| **SCAPs** | Stem Cells From Apical Papilla |
| **SGSH** | Heparan-N-sulfatase |
| **SHEDs** | Stem Cells from Human Exfoliated Deciduous teeth |
| **SRT** | Substrate Reduction Therapy |
| **TALEN** | Transcription activator-like effector nuclease |
| **USCs** | Urine Stem Cells |
| **WT** | Wild-type |
| **ZFN** | Zinc Finger Nucleases |

## Resumo

As doenças lisossomais de sobrecarga (DLS) constituem um grupo de aproximadamente 70 doenças raras, metabólicas e hereditárias, caracterizadas pela acumulação intralisossomal de metabolitos não degradados, como hidratos de carbono, lípidos e proteínas, cuja causa principal é a deficiência ou ausência de atividade de enzimas lisossomais específicas. Por sua vez, estes substratos não degradados ou parcialmente degradados desencadeiam uma diversidade de alterações subcelulares, acabando por se tornar tóxicos para a célula e, consequentemente para todo o organismo, e resultando no aparecimento de doença muitas vezes grave. Neste trabalho, o nosso objetivo foi desenvolver e implementar um novo método para estabelecer modelos *in vitro* de um subgrupo específico das DLS, as Mucopolissacaridoses (MPS). Portanto, uma parte substancial do trabalho descrito nesta tese corresponde a uma extensa caracterização desses mesmos modelos celulares.

Nestas doenças, os substratos acumulados são os Glicosaminoglicanos (GAG). Ao todo, há sete tipos diferentes de MPS, consoante o(s) GAG acumulado(s) e a enzima deficitária. Além disso, alguns destes sete tipos dividem-se, ainda, em vários subtipos.

Em geral, as MPS são doenças multissistémicas, com sintomas em vários sistemas/órgãos do organismo: digestivo, respiratório, pele, visão, audição, etc. Há, no entanto, alguns sistemas, nomeadamente o sistema nervoso central e o sistema esquelético, que são particularmente atingidos nestas doenças Estes dois sistemas têm vindo a tornar-se alvo de maior atenção, uma vez que nenhuma das terapias existentes consegue chegar eficientemente às células que os constituem e, consequentemente, evitar a progressão tantos dos sintomas neurológicos como ósseos.

É neste contexto, que os estudos pré-clínicos *in vitro* se tornam tão vitais, uma vez que permitem o estudo de mecanismos patofisiológicos e o posterior desenvolvimento e validação de eficácia de novas terapias. Uma das abordagens mais frequente passa pela utilização de modelos derivados de células de doentes. Para as MPS, em particular, a maioria dos modelos descritos na literatura são, ou linhas celulares de fibroblastos ou células estaminais pluripotentes induzidas (iPSCs). Contudo, ambos os modelos têm as suas desvantagens associadas. Por um lado, os fibroblastos envolvem o risco de uma "falsa mimetização" dos processos que ocorrem nos dois sistemas referidos. Por outro lado, a geração de células estaminais pluripotentes induzidas (iPSCs, da sigla em inglês *Induced Pluripotent Stem Cells*) é um procedimento bastante dispendioso e com uma série limitações intrínsecas ao próprio procedimento.

Assim, uma solução alternativa que permita contornar estas limitações é a utilização de células estaminais naturalmente presentes em diferentes órgãos e tecidos do organismo humano. Neste trabalho, selecionámos a polpa dentária como fonte natural de células estaminais, por nela se encontrarem as células estaminais da polpa dentária. Estas células, apresentam todas as características frequentemente associadas à estaminalidade celular, nomeadamente, a expressão de uma série de fatores de transcrição específicos e a capacidade inata de diferenciação em outros tipos celulares, bem como de autorrenovação.

Uma vez que as formas mais severas das MPS são pediátricas, considerámos que existia uma população de células estaminais da polpa dentária em particular que poderia evidenciar melhor o que pretendíamos estudar: as células estaminais de dentes decíduos (SHED, do inglês *Stem cells from Human Exfoliated Deciduous teeth*). Estas, para além da elevada taxa de proliferação e da excelente tendência de gerar células esqueléticas e cerebrais, apresentam a vantagem de uma recolha fácil, não requerendo, uma remoção ativa do dente, e, apenas que, no momento da sua queda natural, este seja armazenado nas condições apropriadas.

No entanto, até onde sabemos, esta metodologia nunca tinha sido aplicada a amostras obtidas a partir de doentes com DLS, embora suas vantagens sejam múltiplas e óbvias, especialmente para as formas pediátricas.

Portanto, o objetivo deste trabalho foi estabelecer uma metodologia de cultura celular de SHEDs obtidas a partir de doentes diagnosticados com diferentes tipos de MPS, e caracterizar essas linhas a nível molecular, bioquímico e patofisiológico. Assim, para além do processo de cultura celular, utilizámos também uma diversidade técnicas moleculares, bioquímicas e imunocitoquímicas que possibilitaram a caracterização das linhas celulares estabelecidas.

Primeiro, estabelecemos o protocolo a partir de células estaminais da polpa dentária de crianças saudáveis voluntárias. Assim que as culturas de SHEDs foram estabelecidas e a sua manutenção, armazenamento e passagem foram otimizadas, foi efetuada a confirmação do potencial estaminal destas linhas celulares através de PCR quantitativo em tempo real (qRT-PCR) com marcadores de pluripotência específicos. Com resultados positivos para todos os três marcadores avaliados (Nanog, Oct 3-4 e Sox2), estendemos o "apelo a voluntários" às crianças com MPS e às suas famílias.

A partir daí, recebemos três dentes decíduos de crianças com MPS e conseguimos estabelecer culturas de celulares de SHEDs de todas elas: duas linhas celulares de MPS II e uma de MPS VI. Assim que as culturas foram estabelecidas, confirmámos tratar-se de células estaminais mesenquimais (MSCs, da sigla em inglês *Mesenchymal Stem Cells*) avaliando os níveis

de expressão de vários marcadores relacionados cujos níveis de expressão em MSCs são conhecidos. Posteriormente, estas células foram diferenciadas em diferentes tipos de células, nomeadamente condrócitos, osteócitos, adipócitos e neurónios.

Além disso, foi possível verificar que todas as principais características das MPS já estão presentes nestes modelos celulares: a deficiência da atividade enzimática subjacente; a consequente acumulação de GAG; e, finalmente, a presença de um padrão anormal para a proteína da membrana lisossomal LAMP-1, que se correlaciona com uma distribuição anormal dos lisossomas na célula. De acordo com o que está descrito na literatura, o mesmo não se verifica com os modelos celulares de iPSCs de MPS. Por exemplo o fenótipo de armazenamento, geralmente não é visível em iPSCs; em vez disso, só é evidente depois dessas células serem submetidas a um protocolo de diferenciação. Assim, as vantagens globais do nosso método são bastante óbvias: não só permite um estabelecimento mais rápido e barato de um modelo celular relevante para a doença, mas também tem potencial para recapitular alguns das características celulares e bioquímicas das MPS, que não conseguem ser reproduzidas em modelos de iPSCs.

Em resumo, o trabalho realizado nesta tese, que culminou no estabelecimento de três linhas celulares de SHEDs derivadas de MPS, duas de doentes com MPS II e outra de um doente com MPS VI, constitui *per se* uma inovação total na área das DLS. Estas linhas celulares foram amplamente analisadas quanto ao seu potencial estaminal bem como à presença de características celulares e bioquímicas típicas das MPS e todos os dados reunidos validam a sua utilização como modelo celular para estudar essas patologias em qualquer laboratório.

Por último, consideramos que a abordagem desenvolvida neste trabalho é altamente vantajosa, uma vez que se baseia numa colheita de amostras não invasiva, seguida de um protocolo de cultura celular com elevado custo-benefício, que pode definir uma nova tendência quer para investigar as vias metabólicas celulares que são afetadas nas MPS, quer para testar novas abordagens terapêuticas *in vitro*. Importa ainda referir que, o mesmo princípio aqui utilizado para MPS, pode ser replicado para praticamente qualquer DLS.

**Palavras-chave**: Mucopolissacaridoses; Modelos de Doença; Modelos *in vitro*; Células Estaminais Mesenquimais Dentárias; Células Estaminais de Dentes Decíduos Esfoliados.

# Abstract

Lysosomal Storage Diseases (LSDs) are a group of almost 70 rare metabolic inherited diseases characterized by the intra-lysosomal accumulation of undegraded metabolites, such as carbohydrates, lipids and proteins, mainly due to the inefficient function of specific lysosomal enzymes. As a result, undegraded and/or partially degraded substrates accumulate, triggering a number of subcellular abnormalities. Briefly, it is reasonable to hypothesize that the levels of those undegraded products become toxic for the cell and subsequently for the organism, causing a number of severe and frequently lethal symptoms.

Here we will focus one particular subgroup of LSDs: the Mucopolysaccharidoses (MPSs) and describe how we developed and implemented a novel method to model these pathologies *in vitro*, while extensively characterizing the generated models.

MPSs are a subgroup of LSDs, where the accumulated substrate(s) are Glycosaminoglycans (GAGs). Depending on the GAG that is accumulated, and on the defective enzyme, seven different MPSs exist, some of which may be further divided into additional subtypes. MPSs are multisystemic disorders, with symptoms affecting organs as diverse as the digestive and respiratory traits, skin and eye. Two additional systems severely affected in the majority of those disorders are the skeletal and brain ones. Importantly, however, currently available therapies do not ameliorate brain- and skeletal-related symptoms as both these systems are among the harder ones to get access by those therapies.  In this context, *in vitro* pre-clinical studies in adequate cell models are mandatory to overcome these limitations and study the pathophysiological mechanisms and develop novel and more adequate forms of therapy.

These issues could be overcame by using cell models derived from patients cells, for both purposes: 1) discover new pathology mechanisms and 2) further evaluate the therapeutic effects of novel approaches. The cell models currently available and most commonly used to study MPSs are fibroblasts and iPSCs. However, both have their disadvantages. On the one hand, fibroblasts may not recapitulate disease-relevant features in skeletal and brain systems. On the other hand, iPSCs generation is a time-consuming and extremely expensive protocol with several intrinsic limitations.

An alternative solution for the design of a cell model that could circumvent the existing limitations is the use of naturally-occurring stem cells. In this study, we chose as our stem cell source the dental pulp. Inside this tissue, we may find the so-called dental pulp stem cells. These cells have all the classical features of stem cells, namely the expression of a number of specific transcription factors, differentiation capacity, and self-renewal. Taking into account

that the most severe forms of MPSs are pediatric, there is one particular population of stem cells in the dental pulp that can fit better in the purpose of our study: Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs). Besides, the high proliferation rate and the great tendency to generate both skeletal and brain cells, SHEDs collection does not require the active removal of teeth, only their natural fall.

Nevertheless, to the best of our knowledge, this sort of technology had never been applied to samples obtained from LSD patients even though its advantages are multiple and obvious, especially for the pediatric forms.

Our goal in this work was to establish a method for SHEDs cell culture *in house*, in order to isolate that kind of cells from patients suffering form different MPS disorders and characterize them at molecular, biochemical and pathophysiology levels. Thus, besides the whole cell culture process, a diversity of molecular, biochemical and immunocytochemical techniques were used to characterize these cell lines correctly.

First, we established the whole method for SHED cell culture with samples obtained from volunteer healthy children. As soon as the establishment of primary SHED cell cultures, their maintenance, storage and passage were optimized, we moved on to confirm of the stemness potential of the established SHED cell lines by quantitative Real-Time PCR (qRT-PCR) with specific pluripotency markers. Having positive results for all the three assessed markers (Nanog, Oct3/4 and Sox2), our call for volunteers was extended to MPS children and their families.

From then on, we have received three different deciduous teeth from MPS-affected children, and succeed in establishing SHED cell cultures from all of them: two MPS II cell lines and one MPS VI. As soon as the cultures were established, we validated their mesenchymal stem cell (MSC) identity by assessing the expression levels of a number of MSC-related markers. Additionally, we have also promoted their differentiation into different cell types, namely chondrocytes, osteocytes, adipocytes and neurons.

Furthermore, it was possible to verify that all major MPS disease hallmarks are already detectable in our currently established SHED cell models: the underlying enzymatic activity deficiency; the consequent accumulation of GAGs; and, finally, the presence of an abnormal LAMP-1 staining pattern, which correlates with altered lysosomal positioning. The same, however, does not happen with iPSC-derived MPS cell models, as it has been extensively demonstrated in the literature. The storage phenotype, for example, is usually not visible in iPSC; instead, it is only evident after those cells are subjected to a differentiation protocol.

Thus, the overall advantages of our model are quite obvious: not only does it allow for a faster and cheaper establishment of a disease-relevant cell model, but also holds potential to recapitulate some of the hallmark MPS features, which fail to be reproduced in non-differentiated iPSC models for the same disorder.

Overall, the work performed in this thesis, which culminated in the establishment of three MPS-derived SHED cell lines, two from unrelated MPS II patients, and another from an MPS VI patient, is already a total innovation in the field. Those cells were extensively analyzed for their stemness potential, as well as for the presence of several disease-relevant features and all the data we gathered so far, supports the assumption that they represent a promising model to study these pathologies in any lab with standard cell culture conditions.

Ultimately, we consider this an extremely advantageous approach as it relies on a non-invasive sample collection method, followed by a highly cost-effective cell culture protocol, which may actually, set a new trend not only to investigate the cellular/gene expression changes that occur in MPSs, but also to test novel therapeutic options in vitro. It is also worth mentioning that the same principle, which was used here for MPS, may virtually apply to any LSD.

**Keywords:** Mucopolysaccharidoses; Disease Modeling; *in vitro* Models; Dental Mesenchymal Stem Cells (DMSC); Stem cells from Human Exfoliated Deciduous teeth (SHED)

Aims

## Aims

Considering the limitations presented by the currently available cell models to study skeletal and neuronal systems involvement in Mucopolysaccharidosis, and the need for time- and cost-effective ways to generate novel ones, the main goals of this work were:

- Establishment of a protocol for primary cell culture of Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) *in house*;
- Collection and isolation of both control- and MPS patient-derived SHEDs;
- Confirmation of the stemness potential of the established SHED cell lines, namely by:
    - Quantitiative analysis of the expression of several pluripotency markers;
    - *In vitro* multilineage differentiation into cells from three independent germ layers;
    - Laboratorial confirmation of additional criteria that may allow us to define the established SHED cell lines as Mesenchymal Stem Cells (MSC), namely by evaluation of specific surface antigen expression.
- Assessment of the LSD-associated subcellular phenotype(s) in the established MPS patient-derived SHED, namely by:
    - Molecular and biochemical confirmation of the disease-causing enzymatic defect(s) in each established cell line;
    - Measurement of enzyme activity and GAGs accumulation;
    - LAMP-1 staining.
- Differentiation of the established MPS SHED cell lines into disease-relevant cell types according to the following rationale:
    - Those/SHEDs derived from MPS patients with marked neurodegeneration or obvious neurological phenotypes, into mixed neuronal and astrocyte cell cultures;
    - Those/SHEDs derived from MPS patients with a severe skeletal phenotype or multisystemic disease, into chondrocytes and osteocytes.

# Introduction

# Introduction

## 1. Lysosomal Storage Diseases

### 1.1. The lysosome and lysosomal enzymes

Many components and organelles constitute a viable cell, each one with a specific and significant function(s); the degradation of cell debris and cell metabolism products is achieved by one of the smallest ones: the lysosome.

In order to fulfil their degradative function, lysosomes harbour approximately 60 acidic hydrolases, which are ultimately responsible for the degradation of substrates such as proteins, lipids, carbohydrates, and nucleic acids [1,2]. Nowadays, however, the lysosomes have been shown to have enormous and essential functions such as nutrient sensing, plasma membrane repair, calcium signaling, amino acids and ions homeostasis, trafficking of vesicles, interactions with other organelles among many others, highlighted in Figure 1 [3–5].

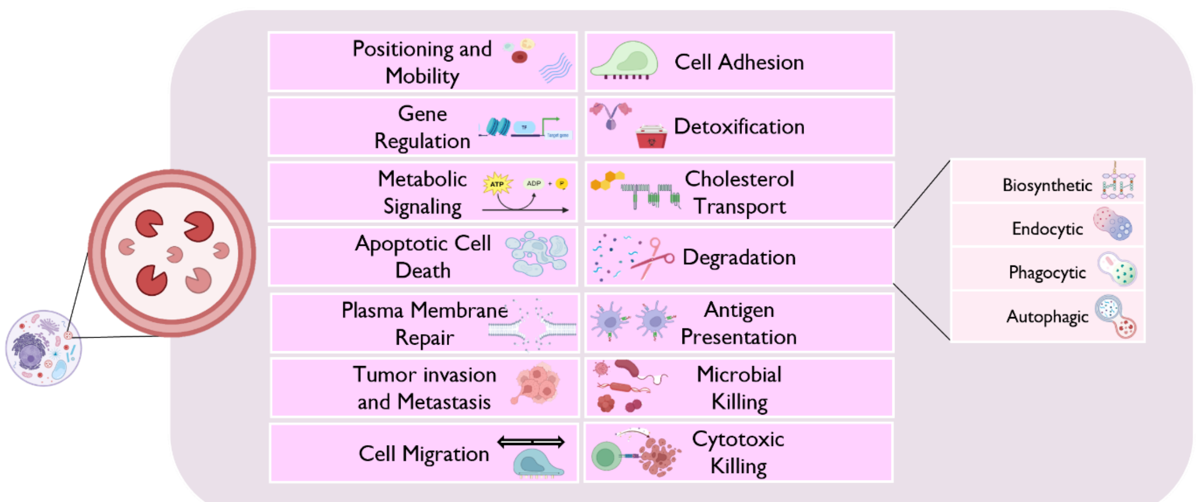


***Figure 1*** - *Essential functions of the lysosome (adapted from Saftig et al., 2009* [277], *created from biorender.com).*

To reach their final destination, lysosomal enzymes need to undergo several post-translational modifications, that will allow their proper sorting and delivery. Briefly, still in the endoplasmic reticulum (ER), where their synthesis occurs, lysosomal enzymes suffer a glycosylation process. However, it is in the Golgi apparatus that the most significant modifications take place. The major modification is the generation of the so-called mannose-6-phosphate (M6P) marker. This is a crucial step for most lysosomal enzymes since it is through proper recognition of this marker by specific M6P receptors that exist in the *trans* face of the Golgi, that those proteins are correctly sorted to the endosome/lysosomal

complex. That sorting relies on clathrin-coated vesicles, which are responsible to transport the cargo to the endolysosomal complex [6]. In addition, this signal also allows for enzyme recycling and recovery since a part of the enzymes is secreted to the blood circulation and may enter back to the cell by M6P-mediated endocytosis [7][8]. Once in the lysosome, the enzymes should be capable of completing their cleavage function. However, when one or more lysosomal enzymes is deficient or absent it fails to complete its function causing its substrates to accumulate, and generating a storage phenotype. Usually, this sort of enzyme dysfunction is caused by genetic mutations in any of the gene(s) that encode for that particular protein and/or participate in their transport to the lysosome. However, in some cases, another protein (enzyme modifier or activator) that is required for optimal hydrolase activity can be defective or absent and a few LSDs are caused by defects in integral lysosomal membrane proteins [9]. Therefore, we can designate LSDs as inherited errors of metabolism in which the function of the lysosome is compromised.

## 1.2. The Lysosomal Storage Disorders group

Altogether, the disorders, which are characterized by substrate accumulation constitute a large group of rare, monogenic, and inherited diseases named Lysosomal Storage Diseases (LSDs). This group comprehends around 70 disorders being almost all characterized by a recessive autosomal pattern of inheritance. Currently, only three exceptions are known, all of them X-linked.

Classically, LSDs are classified into different subgroups depending on the substrate that is accumulated (Table 1) [10]. According to that classification, we can distinguish five major groups of LSDs: Sphingolipidoses (those which accumulate sphingolipids), Mucopolysaccharidoses (those which accumulate mucopolysaccharides, more often designated glycosaminoglicans, GAGs), Oligosaccharidoses (those which accumulate oligosaccharides), Sialic Acid disorders (those which accumulate sialic acid), and Mucolipidoses (which accumulate a number of different substrates, namely mucopolysaccharides, sphingolipids, and glycolipids). But not all LSDs fit into this traditional classification. That is why we can usually find (at least) two extra categories in most of the tables where these disorders are listed: the so-called Neuronal Ceroid Lipofuscinoses (NCLs) and a general category coined Miscellaneous (whose disorders may accumulate substrates as diverse as polysaccharides and amino acids) [11]. There is, however, an obvious link between the majority of the referred disorders: the neuronal storage of undegraded or partially degraded substances, with subsequent cell death in the brain. Accumulation within this system results in a panoply of symptoms including neurocognitive decline, blindness, seizures and, ultimately, premature

death. Still, not every LSD shows an obvious Central Nervous System (CNS) involvement. Some LSDs present in a much more multisystemic way and, for some, the milder forms may actually lack neurological symptoms. Symptoms like hepatosplenomegaly, cardiomyopathy, fibroelastosis, dysostosis multiplex, and cervical spinal cord strangulation are often part of the LSD phenotype, and may be the only clinical manifestations in a number of patients [12].

***Table 1*** *- Different LSDs types and protein/gene associated.*

| Group of Diseases | Diseases | Protein | Gene |
| --- | --- | --- | --- |
| Sphingolipidoses (Accumulation of sphingolupids) | Gaucher Disease | Glucocerebrosidase | *GBA* |
| | Fabry Disease | α-galactosidase A | *GLA* |
| | Niemann-Pick A/B | Acid-Sphingomyelinase | *SMPD1* |
| | Niemann-Pick C | N-acetyl-galactosaminidase | *NAGA* |
| Oligosaccharidoses (Accumulation of oligosaccharides) | Schindler Disease | N-acetyl-galactosaminidase | *AGA* |
| | Fucosidosis | Fucosidase | *FUCA1* |
| | Aspartylglucosaminuria | Aspartylglucosaminidase | *AGA* |
| | Alpha-mannosidosis | α-mannosidase | *NEU1* |
| Mucolipidoses (Accumulation of mucopolysaccharides, sphingolipids, and glycolipids) | Mucolipidosis I or Sialidosis | α -neuraminidase | *NEU1* |
| | Mucolipidosis II or I-cell disease | N-*acetylglucosamine-1-phosphotransferase* | *GNPTAB* |
| | Mucolipidosis III or Pseudo-Hurler-Polydystrophy | N-*acetylglucosamine-1-phosphotransferase* | *GNPTG* |
| | Mucolipidosis IV | Mucolipin-I | *MCOLN1* |
| Miscellaneous (Accumulation of polysaccharides and amino acids) | Pompe Disease | α-glucosidase | *GAA* |
| | Danon Disease | LAMP-2 | *LAMP2* |
| | Cystinosis | Cystinosis | *CTNS* |
| Sialic Acid Disorders (Accumulation of Sialic Acid) | Galactosidosis | Cathepsin A | *CTSA* |
| | Salla Disease | Sialin | *SLC17A5* |
| | Sialuria | Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetyllmannossamine kinase | *GNE* |
| Neuronal Ceroid Lipofuscinoses | Infantile NCL | Palmytol protein thiosterase peptidase-I | *CLN/PPT1* |
| | Late Infantile NCL | Tripeptidyl peptidase-I | *CLN2/TPP1* |
| | Juvenile NCL | CLN3 | *CLN3* |
| | Congenital NCL | Cathepsin D | *CLN10/CTSD* |
| Mucopolysaccharidoses (Accumulation of GAGs or Mucopolysaccharides) | MPS I or Hurler/Scheie Sundrome | α-L-iduronidase | *IDUA* |
| | MPS II or Hunter Syndrome | Iduronate-2-sulfatase | *IDS* |
| | MPS IIIA or Sanfilippo Syndrome type A | Heparan-N-sulfatase | *SGSH* |
| | MPS IIIB or Sanfilippo Syndrome type B | N-acetylglycosaminidase | *NAGLU* |

| | MPS IIIC or Sanfilippo Syndrome type C | Acetyl-CoA glucosamine N-acetyltransferase | *HGSNAT* |
|---|---|---|---|
| | MPS IIID or Sanfilippo Syndrome type D | N-acetyl-glucosamine-6-sulfatase | *GNS* |
| | MPS IVA or Morqui Syndrome type A | N-acetyl-galactosamine-6-sulfate sulfatase | *GALNS* |
| | MPS IVB or Morquio Syndrome type B | β-galactosidase | *GLB1* |
| | MPS VI or Maroteaux-Lamy Syndrome | Arylsulfatase B | *ARSB* |
| | MPS VII or Sly Syndrome | β-glucuronidase | *GUSB* |
| | MPS IX or Natowicz Syndrome | Hyaluronidase | *HYAL1* |

In general, the clinical manifestations depend on the substrate accumulated and on the site where that accumulation occurs. Furthermore, depending on the specific function of the enzyme, which is either missing or dysfunctional, and on its level of deficiency, storage may accumulate at different rates, causing the disease progression to be significantly different [12].

Generically, LSDs are rare diseases. Nevertheless, when considered as a whole, their prevalence may be as high as 1 in 5.000 [10]. Depending on the group and/or subgroup of diseases, there are differences in the severity of symptoms, rate of progression, and organs/systems affected. Still, regardless of their overall severity, LSDs are characterized by a relentless progression of symptoms and no cure is yet known for any of these disorders. There are, however, four different approaches, which have been explored for a number of them and some of them have actually reached the clinic: Enzyme Replacement Therapy (ERT) [13]; Hematopoietic Stem Cells Transplantation (HSCT) [13]; Substrate Reduction Therapy (SRT) [10,13] and Chaperone Therapy [13,14]. It should be noticed, however, that these therapies are only available for a restrict number of LSDs and, even in the cases where a therapeutic option is available, it may fail to address all of the disease's symptoms, as it will extensively discuss.

The most widely used therapeutic approach in the field is also the first one to have been developed: ERT. Briefly, ERT relies on a very simple principle: if LSDs are caused by an enzyme deficiency, one may overcome them by simply giving the enzyme that is missing to the patients who suffer from its dysfunction. Easier said than done, but still, a number of recombinant enzymes are now available in the market and being used by different LSD patients worldwide [15]. Those ERT formulations are administrated intravenously in a periodic manner. Briefly, the recombinant enzyme gets internalized into the cells by the so-called M6P receptors and reaches the lysosomes through the M6P, where it may fulfill its function. The existence of M6P

receptors within the plasma membrane also allows for subcellular cross correction. Meaning: the recombinant enzyme may move from one cell to the next one, thus maximizing its therapeutic effect [15]. However, ERT does hold a series of drawbacks, for instances it may lead to the production of antibodies against the synthetic enzyme. Furthermore, recombinant enzymes do not reach all organs/systems. For example, traditional ERT does not reach the CNS, thus being a real therapeutic option only for non-neurologic diseases or for their non-neurological forms. Despite their limitations, ERTs for Gaucher Disease [16], Fabry Disease [17], Acid Lipase Deficiency [18], Neuronal Ceroid lipofuscinosis type 2 [19], Niemann-Pick disease type A/B [20], Alpha-Mannosidosis [21] , and MPS I, II, IV, VI, and VII [22] are, nowadays, a reality and numerous patients have benefited from them over the last decades. Additional clinical trials with novel enzymes and alternative delivery routes are also ongoing [23]. Overall, ERT is not a cure, but it does significantly increase enzyme activity in many disorders, thus improving their associated clinical symptoms [24].

Another therapeutic approach for LSDs, which has been around for a few decades now, is HSCT [25]. Briefly, we can distinguish 3 types of HSCT: allogenic (when the transplanted cells are derived from a healthy and fully-matched donor); syngeneic (when the transplanted cells are derived from an identical twin); and autologous (when the transplanted cells are derived from the patient before the procedure). While allogeneic HSCT is the standard of care these days for a few LSDs, either syngeneic or autologous transplants are virtually better options, as they work around some of the acute complications associated with HSCT such as veno-occlusive disease of the liver, acute and chronic graft *versus* host disease, and opportunistic infectious conditions. In those two cases, however, the cells which are collected need to be genetically modified *ex vivo* to a normal function. Currently, those approaches are under clinical trial for a few LSDs [26–30]. Regardless of the HSCT type, in terms of procedure, its principle is simple: first, the patient needs to receive some type of therapy that will inhibit the immune system (to prevent rejection); then the modified cells are injected in the patient. Due to their stemness potential, the graft cells, which are capable of synthesizing functional target enzymes, will rapidly proliferate and differentiate providing a natural, endogenous source of the enzyme, which was previously missing [31].

Still, this approach does not seem to be effective for a number of LSDs where, in theory, it should work [32]. There are, however, a few diseases for which this procedure is highly recommended and does show exceptional results if performed soon enough. That is the case of one particular form of MPS: the Hurler syndrome (the severe forms of MPS I). Transplantation is still considered the "standard of care" for patients suffering from that

syndrome. Nevertheless, this procedure in only effective when performed at the very initial stages of the disorder. In fact, it has only been shown to enhance the cognitive function in patients with less than 9 months [10,25,33].

Even though Hurler syndrome seems to be the perfect example on the success of HSCT, there are some general considerations we can draw for other LSDs to which may apply. Usually, visceral symptoms can be improved, whereas skeletal lesions remain relatively unaffected. The effect on neurologic symptoms varies. Still, HSCT remains a viable treatment option in those LSDs where data supportive of disease stabilization or amelioration is known (reviewed in [34]).

But there are two other, more recent approaches, which may be used to overcome the LSD-associated pathology. The first one is SRT, with licensed products available for Gaucher disease and Niemann-Pick Type C. Again, its rationale is quite straightforward: it promotes an overall reduction of the accumulated substrate(s) by inhibiting its biosynthesis, thus ameliorating the associated phenotype(s). Unlike ERT, the presently available substrate reduction drugs are orally administrated, and some of them have the ability to cross Blood-Brain Barrier (BBB) achieving an effect on CNS [20]. Still, this option has a slower onset efficacy, and so far, it is restricted to sphingolipidosis. The conjugation of SRT with other therapies may significantly improve the treatment of LSD [5],[21].

Finally, there is also the so-called chaperone therapy. Pharmacological chaperones are small molecules defined by their ability to help a protein fold correctly [37]. By doing so, those molecules will help their target protein escape proteasomal degradation and reach an adequate subcellular destination, where it can exert its function. Basically, this molecule binds to the misfolded protein in the ER forming a stable complex that prevents the misfolding. When the complex arrives to the lysosome, dissociation occurs. As a result, a functional or partially functional protein gets internalized into that organelle, where it can exert its activity [14]. It is worth mentioning that this sort of therapeutic approach may only work for disease-causing missense mutations. So far, Fabry disease (one of the most common LSDs worldwide) is the only LSD with an approved chaperone therapy that is currently being used in the clinic for a significant number of Fabry disease patients, all harboring missense mutations that cause misfolding of $\alpha$-galactosidase, and has been shown to improve the associated cardiac and renal symptoms [38,39]. And, while no other chaperone molecule has reached the clinic so far, several studies are being performed in other LSDs (e.g.: [40–44]).

## 1.3. Mucopolysaccharidoses (MPSs)

Among the LSDs in need for better and more effective therapeutic options are the Mucopolysaccharidoses (MPSs). The MPSs subgroup includes seven different disease types, all of them accumulating GAGs as the primary substrate. An overview of each individual disorder is described below.

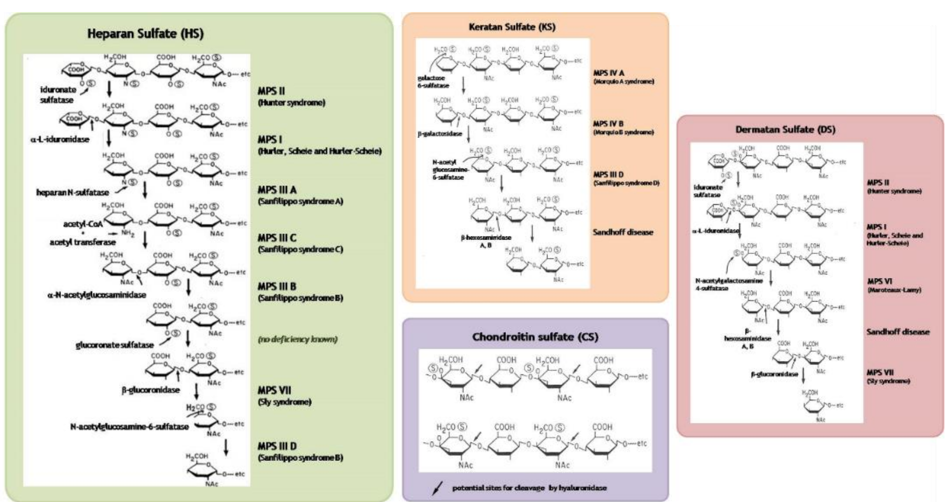


***Figure 2*** *- Degradation of Glycosaminoglycans. Stepwise degradation of heparan sulfate (HS), keratan sulfate (KS), dermatan sulfate (DS) and chondroitin sulfate. For HS, KS and DS, both the enzymes involved in each reaction and their associated deficiency diseases are indicated. Concerning the degradation of CS, arrows show potential sites for cleavage by hyaluronidase. The oligosaccharides are further hydrolyzed by the stepwise action of N-acetylgalactosamine 4-sulfatase or 6-sulfatase, β-hexosaminidase A or B, and β-glucuronidase.*

MPS I is one of the most common forms of MPS and the first MPS type treated with ERT (available since 2003) [45]. At a clinical level, MPS I may be divided into three subtypes: Hurler (OMIM #607014), Hurler-Scheie (OMIM #607015), and Scheie (OMIM #607016) depending on the disease severity [46]. Hurler syndrome is the most severe form of them all and Scheie is the mildest, with Hurler/Scheie being a somehow intermediate phenotype, but in general, type I has an incidence of 0.11 [47] to 3.62 [48] per 100.000 live births (reviewed in [49]). As the majority of LSDs, MPS I is characterized by a progressive pattern that includes several stages of clinical manifestations. In this multisystemic disease during the first 6 months of life, the children present symptoms such as coarse facies, hepatosplenomegaly, and upper airway obstructions that usually evolve to more specific and severe symptoms associated to the constant increase in the accumulation of GAGs in the soft tissues, bones, spleen and liver and the most severe cases in the brain. Overall, dysostosis multiplex is considered the most common clinical symptom of MPS I [50]. Regardless of the clinical presentation, *IDUA* is the affected gene in this disorder. Mutations in this gene, which encodes for α-L-iduronidase (IDUA; E.C. 3.2.1.76), lead to an enzyme deficiency that ultimately results in heparan and dermatan sulfate (HS and DS, respectively) accumulation [51]. To date, 359 disease-causing

mutations ([52]) are identified for this gene and currently, there are two possible forms of therapeutics: ERT and HSCT, which is only used in the most severe form of the disease and, preferably in the first years of life [53]. Regarding ERT, there is only one recombinant enzyme approved for MPS I: laronidase (Aldurazyme®, Genzyme). As every other ERT, this recombinant enzyme is injected into the blood circulation, which leads to the correction of the enzyme deficiency in various organs and tissues, except the brain, once it does not cross the BBB [54,55].

MPS II (OMIM #309900), or Hunter syndrome, is the only X-linked MPS disease; all the other MPSs are autosomal. Thus, in the Hunter syndrome, males are the most affected with a prevalence of 0.1 [56] to 2.16 [57] in 100 000 live births (reviewed in [49]). Two forms of the disease may be distinguished: neuronopathic and non-neuronopathic, being the most severe the CNS-associated [58]. Regarding clinical manifestations, the skeletal, cardiac and respiratory systems are the ones mostly affected. In the most severe cases, adding up to the symptoms affecting the previously referred systems, there is also an involvement of the CNS. Usually, for the neuronopathic form, the average life expectancy is around 10-15 years of age, while the individuals who suffer from the attenuated one may live beyond 50 years [59]. Regardless of the subtype, MPS II is caused by mutations in the *IDS* gene, which encodes the enzyme iduronate 2-sulfatase (IDS; EC 3.1.6.13). The *IDS* gene is split into 9 exons, spanning approximately 24 kb [60]. There are 795 mutations identified to date, which may cause this syndrome ([52]). The IDS deficiency leads to the accumulation of two substrates: HS and DS. Regarding MPS II therapeutics, ERT with idursulfase (Elaprase®, Shire) is the first choice for patients with this condition [61].

MPS type III , also known as Sanfilippo syndrome, may be subdivided into 4 subtypes: III A (OMIM #252900), III B (OMIM #252920), III C (OMIM #252930), and III D (OMIM #252940). Each particular subtype is associated to a unique enzymatic defect: MPS IIIA is caused by the deficiency of the enzyme Heparan-N-sulfatase (SGSH, EC 3.10.1.1); MPS IIIB, by its turns is caused by defects in the enzyme N-acetylglucosaminidase (NAGLU, EC 3.2.1.50); in MPS IIIC the protein involved is the transmembrane enzyme, Acetyl-CoA glucosamine N-acetyltransferase (HGSNAT, EC 2.3.1.78) and, finally, the MPS IIID is caused by defects in N-acetyl-glucosamine-6-sulfatase (GNS, EC 3.1.6.14). Regardless of the enzymatic defect itself, all of them are associated with a severe deterioration of neurological function [62], which results in a number of clinical symptoms either directly or indirectly related to a CNS dysfunction, such as behavior problems, sleep disturbances, hearing impairment, development regression, recurrent infections in the respiratory tract, and facial dysmorphology [63][64].

The general prevalence is 0.06 [65] to 1.89 [66] in 100.000 live births (reviewed in [49]), with types A and B being more common for most populations than C and D [67]. Regardless of the affected genes, the stored substrate is always HS.

Various disease-causing mutations were already identified for the different forms of MPS III [68]: in the case of *SGSH* gene (with a total of 8 exons and associated with type IIIA), 163 mutations have already been identified; in type IIIB, 215 mutations have already been identified in any of the 6 exons that constitute the *NAGLU* gene, or their surrounding intronic sequences; in the *HGSNAT* gene, 93 mutations along the 18 exons and their respective introns are known to cause the deficiency observed in type IIIC. Finally, in type IIID, where the *GNS* gene (which spans 14 exons) is mutated, only 25 mutations were identified ([52]). Unfortunately, there is no approved treatment for these neurologic diseases. On the one hand, while it has already been attempted by several different teams, HSCT has proven virtually no benefit over the neurocognitive symptoms [69–73]. On the other hand, ERT is hard to apply, once classically formulated enzymes do not penetrate the CNS. Moreover, in the case of MPS IIIC, for example, ERT is not an option, once the deficient enzyme is a transmembrane protein.

There are, however teams attempting brain-specific delivery of both ERT and chemical compounds for MPS type III. In general, there are three strategies to increase the delivery (reviewed in [74]): enzymatic modulation, route(s) of administration [75–77], and increase of enzyme dosage. In addition, cellular and genetic therapies represent approaches that have gained importance when it comes to BBB delivery (reviewed in [78]). Targeting brain cells through enzymatic modulation consists of the combination of the enzyme with protein/peptides than can facilitate BBB crossing (reviewed in [79,80]). In the cellular and genetic therapies field, among other possibilities, gene therapy with the use of adeno-associated virus has been stealing a lot of attention with extensive works to reach the BBB showing the intended effect [76,81–83]. Besides the modifications above referred, SRT constitutes also an alternative to get through the BBB. [84–86] The development of a valuable treatment has reached very high levels of need so that regulatory initiatives to support the development of a possible treatment are commonly found [62,68,87,88].

There are two different forms of MPS IV, each one caused by a single enzymatic defect: N-acetyl-galactosamine-6-sulfatase (GALNS; E.C. 3.1.6.4) deficiency underlies MPS IVA (OMIM #253000) while beta-galactosidase (BGAL; E.C. 3.2.1.23) defects cause MPS IVB (OMIM #253010). The involved genes are *GALNS* and *GLB1*, respectively [89,90]. MPS IV, or Morquio Syndrome, has an incidence of 0.07 [65,91] to 3.62 [48] in 100 000 live births (reviewed in [49]) . Unlike MPS III, which is almost exclusively a neurological syndrome, the skeleton is the

main affected system in MPS IV, with the substrate accumulating predominantly in the cartilage and bones. Consequently, the major clinical manifestations observed are bone deformations, short stature, and mobility alterations [92]. In both cases, keratan sulfate (KS) and chondroitin-6-sulfate (C6S) are the accumulated substrates. So far, 467 mutations have been described in the *GALNS* gene [52], composed of 14 exons, all associated with MPS IVA [93][94]. Concerning type IVB, 263 *GLB1* mutations are known to cause this disorder. The only approved treatment for MPS IV is elosulfase alfa (Vimizim®; BioMarin Pharmaceutical Inc.) that is used MPS IVA patients. All other options are symptomatic and mostly consist in surgical approaches to prevent spinal cord damage or other skeleton issues, for example, spinal decompression surgery [95].

Yet another form of MPS, usually coined as Maroteaux-Lamy Syndrome, is MPS type VI (OMIM #253220). At least 242 mutations in the *ARSB* gene (which spans 8 exons) are known ([52]) to cause this disorder The estimated frequency for this disorder is 0.0132 [96] 7.85 [48] in 100 000 live births (reviewed in [49]). Even though being a multisystemic condition, MPS VI does not affect intelligence, and, like Morquio, the skeleton is the most affected system [97]. Thus, the clinical manifestations are very similar to those described above including short stature, low body weight and impaired pulmonary and motor functions [98]. To counteract the DS storage promoted by the deficiency of Arylsulfatase B (ARSB; EC 3.1.6.12) activity, galsulfase (Naglazyme®, BioMarin Pharmaceutical Inc) is the drug approved and currently employed in patients [99].

MPS type VII (OMIM #253220) or Sly syndrome occurs with an estimated frequency of 0,02 [65,100–102] to 0,29 [56] per 100.000 live births (reviewed in [49]). Several systems/organs are involved in this disease with clinical features affecting organs as diverse as the eyes, lungs, heart, musculoskeletal, spleen, etc. Thus, the most common symptoms are described as coarse facial features, increased of cranial circumference, reduced of pulmonary function, obstructive airway disease, dystosis multiplex, decrease of mobility, joint contractures, abdominal abnormalities, short stature and hepatomegaly/splenomegaly. There may also be a neurological involvement as testified by recurrent observations of limited vocabulary and mental retardation in several MPS VII patients [103]. Overall, these symptoms are caused by an ubiquous accumulation of several different GAGs, namely DS, HS, and CS, as a consequence of the deficient activity deficiency of β-glucuronidase (GUSB; EC 3.2. 1.31). The *GUSB* gene (12 exons) [104] with 81 mutations identified so far ([52]), is the one affected in this disorder [105]. The approved drug for this pathology is vestronidase alfa (Mepsevii™, Ultragenyx), which is indicated in both pediatric and adult cases [106].

Finally, MPS IX or Natowicz disease (OMIM #601492) is an ultra-rare disorder. The first report was published in 1996, with the described patient presenting a number of clinical manifestations associated to joint and skeletal systems [107]. This disorder is caused by a deficiency of the enzyme hyaluronidase 1 (HYAL1; EC 3.2.1.35) due to mutations in the *HYAL1* gene (3 identified until now [52]), which leads to the accumulation of yet another substrate: hyaluronan. Due to the rareness of the disorder, very few mutations have been reported to date (only 7), and a possible treatment is very challenging [108].

In general, even though the molecular bases and biochemical defects underlying MPS diseases are well defined, knowledge is still lacking on the pathophysiological mechanisms that actually trigger the appearance of different symptoms in the different organs and systems. And, even though much has been learnt over the last decades, from the study of individual patients and, particularly, from the generation and extensive characterization of bona fide *in vivo* models, truth is we haven't still fully understood the whole physiological cascade, which underlies some of MPSs' most challenging phenotypes, namely those which affect the CNS. And this is particularly relevant since no therapeutic exists to ameliorate them. Still, finding an *in vitro* model that could recapitulate the disease-relevant features is also challenging once live neurons are inaccessible cells. Indeed, for almost a century, patient-derived fibroblasts were gold standard for in vitro studies in MPSs, as in all other LSDs. These cells were relatively easy to access, since a simple skin biopsy would be enough to obtain them and remarkably, they did display the hallmark cellular phenotype that actually coined these diseases as "storage" disorders: the presence of undegraded or partially degraded substrates. Nevertheless, fibroblasts may also fail to recapitulate disease-relevant features, which are more evident in other particular cell types, of higher pathological significance such as neurons. A viable option is to generate the neurons from a patient-derived cell line, which involves extracting the cell from the patient and differentiating it into neuronal cells. Indeed, there are two possible ways to do this process: to use induced pluripotent stem cells (iPSCs) or Mesenchymal Stem Cells (MSCs) obtained from the patient.

## 2. *In vitro* models

### 2.1. Modeling genetic disorders

The establishment and analysis of human cell cultures concedes to science the possibility of investigating, in a progressive way, every detail of the human body (either disease- or non-disease-affected). This technique, under restricted conditions, has the purpose of mimicking every single mechanism that cells present *in vivo*, in a controlled environment by ensuring their

correct proliferation and survival rate *in vitro* [109]. In fact *in vitro* cultures may allow us to model essential life events such as diseases, ageing, biological barriers, and interactions with pathogens, being considered a fundamental tool in fields such as biology and medicine [95], [110]. Modeling diseases from cell culture can work as a way to develop and evaluate new therapies and discover new biomarkers, which are extremely relevant not only for the diagnosis but also for the prognosis of a given disease. In addition, cell culture appears as an alternative to animal models, being an important step towards respecting the 3Rs (replacement, reduction and refinement) ideology [111,112]. Genetic diseases, particularly monogenic ones, are among the most interesting ones to study *in vitro*, in different cell models because their causing genetic mutation(s) are usually expressed in the cells extracted from the affected individuals. The comparison between "healthy" and "diseased" cells may then provide valuable clues on the disease pathogenesis, while allowing for drug screenings, genotype-phenotype correlations, etc [113]. In general, the establishment of that sort of cell culture is a process that involves the extraction of patient cells. In the case of LSDs, patient-derived fibroblasts obtained after skin biopsy are the most commonly used approach.

## 2.2. Current MPSs *in vitro* models

In general, even though the molecular bases and biochemical defects underlying MPS diseases are well defined, knowledge is still lacking on the pathophysiological mechanisms that actually trigger the appearance of different symptoms in the different organs and systems. And, even though much has been learnt over the last decades, from the study of individual patients and, particularly, from the generation and extensive characterization of bona fide *in vivo* models, truth is we haven't still fully understood the whole physiological cascade, which underlies some of MPSs' most challenging phenotypes.

### *2.2.1. Fibroblasts*

Patient-derived fibroblasts have been extensively used to study LSDs. These cells were actually the gold standard for *in vitro* studies in LSDs for various decades, and there are several reasons to justify their success. First of all, they were relatively easy to access, since a simple skin biopsy would be enough to obtain them. Furthermore, there are numerous effective protocols for isolation and establishment of primary cell culture [114]. And, remarkably, they did display the hallmark intracellular phenotype that actually coined these diseases as "storage" disorders: the presence of undegraded or partially degraded substrates.

The establishment of those cell lines allowed the scientific community to unveil and catalogue some intrinsic features of those disorders, which were previously unsuspected such

as cell cycle disturbances [115], transcriptomic changes [116], enzyme activity and kinetics [117–120]. They have also greatly contributed to testify the biochemical, molecular and mutational heterogeneity, which characterizes this group of disorders [28]. Additionally, those cell lines have also allowed for the *in vitro* assessment of the potential therapeutic effect of numerous approaches and compounds [121–124]. In fact, the knowledge these cells have helped us achieve over the years, makes it easy to explain why they represent such a great model for those disorders: not only do they recapitulate the primary defects underlying these disorders, but also the storage that results from it and, most probably, many of the pathophysiological cascades that it triggers.

Despite being a successful and reliable model for these genetic diseases, fibroblasts may also fail to recapitulate disease-relevant features, which are only evident in other particular cell types, of higher pathological significance. For example, in the case of MPS diseases where brain and skeleton are severely affected, there is a significant need for disease-relevant cell models that actually mimic any of those two systems/organs for deeper and more accurate pathophysiological study. In fact, these two systems are indeed hard to access and none of the therapeutic options, which is currently available seems to be able to correct the symptoms they develop. Therefore, even to screen for novel or better therapeutic solutions that hold potential to properly reach these targets, it is imperative to find alternative models that allow for the assessment of the drug candidate in its ultimate cellular target [125].

#### *2.2.2. Induced Pluripotent Stem Cells (iPSCs)*

To overcome the major issue of fibroblasts, another cell model recently developed is the resource of induced Pluripotent Stem Cells (iPSCs) technology.

Human iPSC generation in particular started its journey in 2007, when Yamanaka et al. [126] first generated those cells from human somatic fibroblasts using a remarkable method, which relies in the retroviral transduction of 4 independent transcription factors into patients' fibroblasts: Oct3-4; Sox2; Klf4, and c-Myc. Remarkably, the cells that resulted from this experimental setup showed numerous similarities with human embryonic stem cells including morphology, proliferation capacity, gene expression pattern, and *in vitro* differentiation potential. Ever since this original report was published, the search for novel and improved protocols for cells reprogramming advanced at an outstanding pace, with various optimizations being published in order to generate virtually every cell of interest from iPSC of different origins [127].

Over the past few years, *in vitro* models derived from iPSCs have been unraveling some enigmatic aspects of MPSs. In particular, the subtypes that present neurological involvement appear as the ones with the greatest need for additional knowledge and new therapeutic solutions. In general, the studies published so far using iPSC as a tool to model MPS may be divided into four different groups according with their ultimate goals (Figure 3) : (1) those aimed at the generation of MPS-derived iPSCs; (2) those, which aimed at differentiating those iPSCs into relevant cell types (particularly neurons or neuronal precursors) and assessing their disease modeling potential; (3) those whose goal was to use the generated iPSCs or iPSC-derived (neuronal) models as a platform for *in vitro* drug screening of therapeutics; and (4) the one that described the generation of those cells for gene therapy purposes[1].

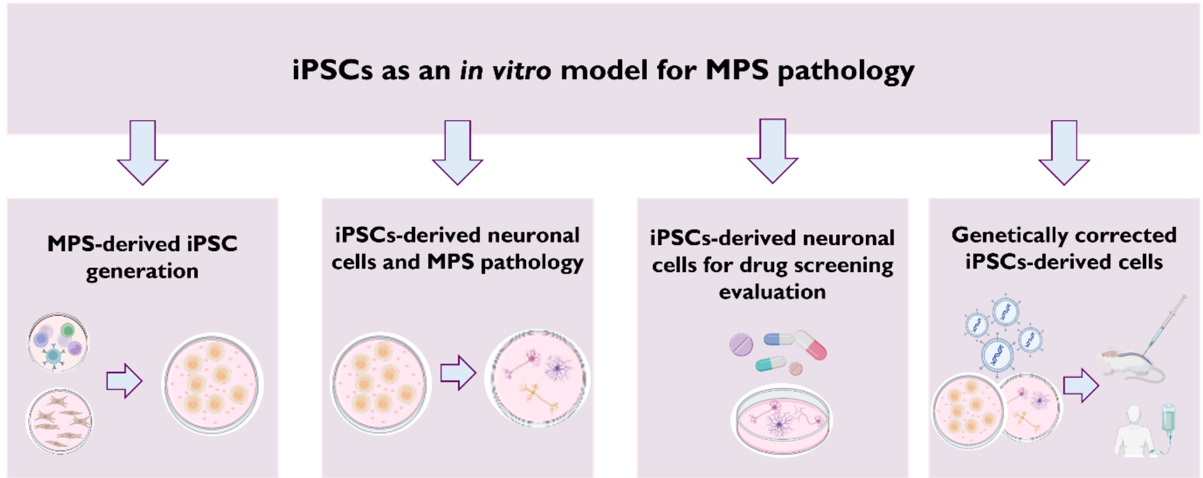


***Figure 3*** *- The four aims possible to achieve with MPS-derived iPSCs in vitro models. (Adapted from biorender.com)*

The first work using the iPSC technology to model MPS diseases was published in 2011 by Thomas Lemonnier and colleagues [128], who reprogrammed fibroblasts from two patients suffering from MPS IIIB into iPSCs. As required for virtually every iPSC generation report, the resulting stem cells were extensively analyzed and characterized. In this particular study the authors confirmed a positive expression of three particular pluripotency markers (SSEA4, Nanog, and TRA-1-60) and the differentiation ability of those cells, thus proving their pluripotency nature. Additionally, the authors have also provided information on the karyotype presented by those cells. This is a relevant assessment whenever a novel iPSC line is generated but it should also be considered later on, when using the same iPSC line after several passages, or after having one particular iPSC cell line in culture for a long period. In

[1] For an extensive review on the works performed so far using iPSC technology to model MPSs, see Annex 1, review paper 1: Carvalho et al., **Neurological disease modeling using Pluripotent and Multipotent Stem Cells: a key step towards understanding and treating Mucopolysaccharidoses** [Under Preparation]

fact, long-term iPSCs culture is known to result in chromosomal abnormalities, changes in gene expression and cellular functions, and even increases the risk of the iPSCs being tumorigenic. As genomic alterations present potential risks in the overall applications of iPSCs, it is crucial to monitor the genomic integrity of iPSCs lines. That is why iPSC karyotype analysis is such an important step on the validation of this type of cell models, and nowadays considered as a routine procedure by all the groups working with iPSC technology.

Thereafter, numerous studies reported the generation of MPS-derived iPSCs, generated both by peripheral blood mononuclear cells [129–132] and fibroblasts [133–135]. In addition, an innovative approach was attempted by Noelia Benetò et al., who generated this type of cells from healthy iPSCs using CRISPR/Cas 9 to generate isogenic mutated lines. By using this innovative gene editing technology, they created human-derived cell lines with the same genetic background, differing only in the gene of interest [136]. These isogenic pairs are powerful tools for understanding gene function. In fact, by circumventing confounding effects of genetic background, they allow for more accurate and reliable genotype-phenotype correlation studies [137].

In general, the iPSCs generated in the majority of those studies were derived from patients who suffered from neurological forms of MPSs, or who presented with at least some CNS-related symptoms. In fact, the majority of the studies published so far was performed in cells derived from severe forms of MPS I and II or from MPS III. Naturally, that neurological involvement could be further explored by differentiating iPSCs into different types of neuronal or pre-neuronal populations. So, many teams that originally reported the generation of MPS-derived iPSC cell lines, focused on their subsequent differentiation into disease-relevant neuronal cells. Overall, their results further highlighted the modeling potential of iPSC-derived cell lines, by showing numerous pathophysiological insights one can get with a few simple cellular assays.

Again, right after the neurodifferentiation protocols were carried out, and before any kind of pathophysiological assay was performed, the generated cell lines, were extensively characterized, usually through the assessment of specific markers. Briefly, when the team's final goal was to develop neuronal stem cells (NSC) or neuroprogenitor cells (NPC), they checked for the levels/expression of neuronal markers such as Nestin, Pax-6, and Sox2. When their goal was to generate astrocytes, they used markers such as Glial Fibrillary Acidic Protein (GFAP). And, finally, when their ultimate goal was to generate active neurons, they checked for MAP2 and Synapsin. So, only when a proper neurodifferentiation was confirmed, did they move on to the analysis of disease-relevant features. For example, the analysis of lysosome-

associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) was a common assessment [128,138,139]. In fact, since these two proteins are major components of the lysosome membrane, checking their expression levels and sub-cellular localization is a simple way to confirm the lysosomal phenotype that arises from the storage lesions. Yet, other organelles have also been analyzed in these MPS-derived iPSC cell models, some of which also did present signs of abnormal function. For example, the first report of Golgi complex impairment in MPS pathology was described precisely in one of these iPSCs cell lines [128] through the analysis of GM130 fluorescence.

Besides the already known MPS-relevant features, also events more related to neuropathology have been investigated in these studies: the effect of HS accumulation on focal adhesions [140]; the global interactions in the neuronal network [141], the abnormal proliferation rates [139] related to the interaction of HS and growth factors [142] and with a lower neurite outgrowth and cell migration [143]; the increases in autophagy, demonstrated by different autophagy markers and ER stress tests [139]. Moreover, some transcriptomic analysis were also performed, highlighting several signaling pathways, which were altered in iPSCs with MPS neuropathology [143].

As we have already referred, the majority of studies using iPSCs to investigate MPS-related pathology are focused on MPS I, II, and III, due to their neurological involvement, and to the well-known impossibility of currently existing drugs to reach the CNS. Still, skeletal involvement in MPSs is also an issue that needs addressing, once the available therapeutics have a narrow effect window on cartilage and bone. That is why, the emergence of *in vitro* models for those two organs is starting to grow. In fact, to best of our knowledge, the first attempt to generate MPS-derived iPSCs for subsequent chondrogenic differentiation, was only published in 2022 by Broeders et al. In that pivotal paper, besides generating iPSCs from MPS VI patients and their respective isogenic controls and subsequently differentiating them into chondrocytes, the authors have also performed a genome-wide mRNA expression analysis, which allowed for a significant increase in the knowledge about the genes, which are up- and downregulated, in cartilage in MPS VI [144].

As already referred, there is a third group of papers using iPSC technology in MPS, whose aim was to use the generated cells as a platform for *in vitro* drug screening. In fact, numerous therapeutics were already tested in those cells: siRNAs against genes responsible for GAGs biosynthesis [145], ERT with recombinant enzymes [146,147], and other compounds that had already shown to ameliorate phenotypic events on other LSDs, such as δ-tocopherol (DT), and hydroxypropyl- β-cyclodextrin (HPBCD) [146,148].

The fourth and final aim we have referred to is the use of these cells in another kind of therapeutic approach: *ex vivo* gene therapy. The proof of concept study was published in 2015, when genetically corrected GFP-labelled NSCs where injected intraventricularly into different neonatal mice populations, either suffering or not from MPS VII. The results of this study showed that it was possible to detect the enzyme activity but only near the injection site, accompanied by a reduction of neuroinflammation [149]. After that first study, others emerged, always confirming that iPSC-based gene therapy was able to improve enzyme activity and reduce some neuropathological events such as glial and astrocyte activation, and/or storage accumulation [150,151].

***Table 2*** - *Works performed in MPSCs using iPSCs technology.*

| Disorder | Affected Gene | Defective Enzyme | Stored Substrate | Subtype | Generation of MPS-derived iPSCs | | | | Drug Screening | *Ex vivo* gene therapy |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Source | iPSC | NPC | Mature neurons | | |
| MPS I | *IDUA* | α-L-iduronidase | DS and HS | Hurler | Fibroblasts | [135,143,152] | [143,152] | | | |
| | | | | | Mouse Embryonic Fibroblasts | [153] | | | | [153] |
| | | | | Hurler/Scheie | Fibroblasts | [152] | [152] | | | |
| | | | | Scheie | Fibroblasts | [132] | | | | |
| | | | | | PBMCs | [152] | [152] | | | |
| MPS II | *IDS* | Iduronate-2-sulfatase | DS and HS | | Fibroblasts | [142,148] | [142,148] | | [148] | |
| | | | | | PBMCs | [129–131,147] | [139,147] | [139,147] | [147] | |
| MPS III | *SGSH* | Sulfamidase | HS | A | Fibroblasts | [134] | | | | |
| | *NAGLU* | α-N-acetylglucosaminidase | | B | Fibroblasts | [128,133,140,146] | [128,133,140,146] | [146] | [146] | |
| | | | | | Mouse Embryonic Fibroblasts | [150,151] | [150,151] | | | [150,151] |
| | *HGSNAT* | N-acetyl-transferase | | C | Fibroblasts | [136,141] | [138,141] | [138,141] | [138,141] | |
| MPS VII | *GUSB* | β-Glucuronidase | DS, HS, and CS | | Mouse Embryonic Fibroblasts | [149] | [149] | | | [149] |

Regardless of its ultimate purpose, in general, the rationale followed in all the studies reviewed so far is the same: first, differentiated cells from patients with the target disease are reprogrammed into iPSCs and, then, differentiated again but into disease-relevant cell lines, thus creating a viable cell model for neuronopathic MPS. This technology, as described above, is undoubtedly contributing to increase the knowledge on the pathophysiology of MPSs with neurological involvement and, consequently, with no treatment available. Nevertheless, while iPSC technology proves to be quite valuable and promising, it also involves some disadvantages. Those positive and negative considerations are recapitulated in the Figure 4.

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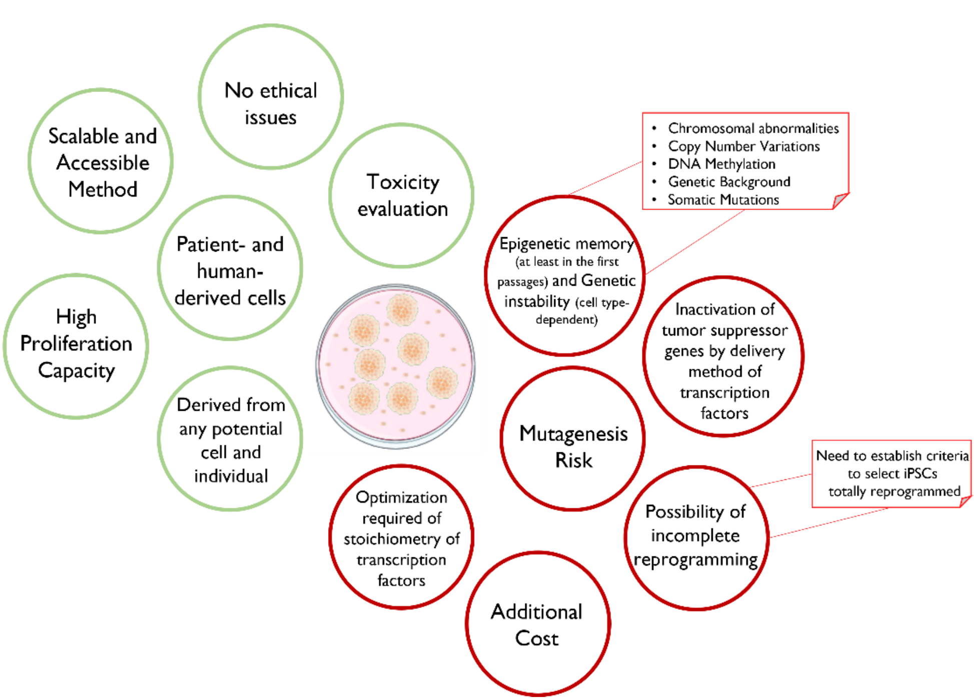


***Figure 4*** *- Advantages (in green circles) and Limitations (in red circles) of iPSCs generation*

That is why, alternative protocols and additional sources of stem cells should also be considered, especially those, which are naturally-occurring. An excellent option would be to take advantage of patients' MSCs, reducing the possibility of errors and avoiding the long, laborious and expensive pluripotency induction phase. In fact, those cells represent a suitable alternative once they can be differentiated into any of the three germ layers: endodermal, mesodermal, and ectodermal, as long as they are cultured in proper media.

## 2.3. Other cells that could recapitulate disease-relevant features

### *2.3.1 Mesenchymal Stem Cells (MSCs)*

MSCs have, like all stem cells, the ability to self-renewal and differentiation into multiple cell lines, ultimately representing different organs and systems of the body. A remarkable characteristic of this type of cell culture in general is the presence of structures known as fibroblast colony-forming units (CFU-F), which can be attributed to their general fibroblast-like morphology [154]. But there are many other criteria a cell has to fulfill to be defined as a MSC [155]. Currently, the minimal criteria are (Figure 5):

(1) Adherent cells with spindle morphology when in standard culture conditions;

(2) Markers in cell surface positive for: CD105, CD73, and CD90; Negative for CD45, CD34, CD14, CD79α, and HLA-DR antigens;

(3) Osteogenic, chondrogenic, and adipogenic differentiation capability *in vitro*.

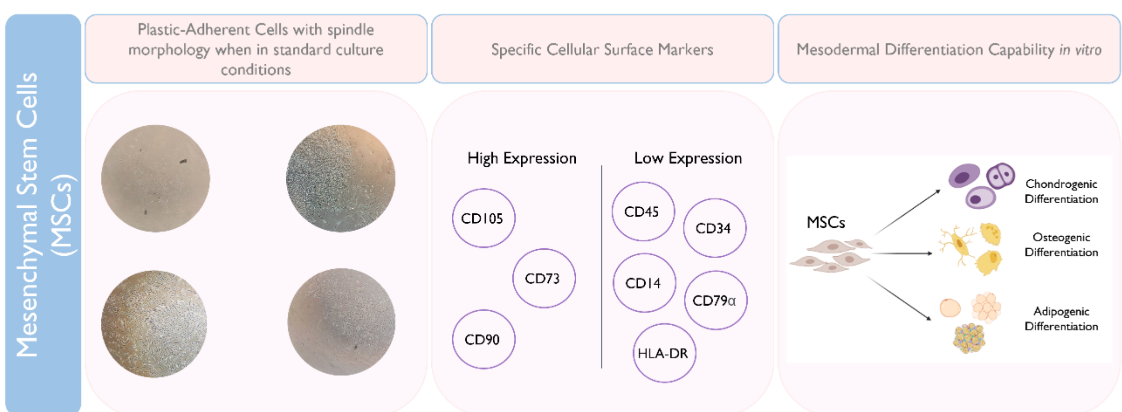


***Figure 5*** *- Minimal Requirements for identification of MSCs (adapted from biorender.com).*

However, these requirements need to be reconsidered since this research area has been in constant growth in the last few years. For example, there is a growing number of markers which appear to be associated with stemness, namely STRO-1, SSEA-1 and -4, CD271, and CD146 [156]. Furthermore, numerous authors support the idea that MSCs should be able to differentiate into more than the traditionally required cell types (adipocytes, osteocytes and chondrocytes), as they do not account for the 3 independent germ layers. In fact, according to those authors, for a certain cell to be classified as MSC, it should be able to differentiate into cells from any of the 3 germ layers, depending on culture conditions: mesodermal (e.g.: osteogenic, adipogenic and chondrogenic); ectodermal (e.g. neurogenic differentiation) and endodermal (e.g.: pancreatic and liver cell differentiation).

Naturally-occurring MSCs can be found in many different tissues such as the umbilical cord, adipose tissue, menstrual blood, bone marrow, dental tissue, placenta, peripheral blood,

ligaments, etc (Figure 6). However, the primary source considered nowadays is bone marrow, even though it does present some disadvantages, which will be further explored later on. As referred above, homeostasis maintenance and specialized differentiation are the functions of these types of cells [156].

Despite having a well-characterized source and being the most commonly used, Bone Marrow Mesenchymal Stem Cells present some disadvantages, which may justify the need to explore different sources of MSCs. Among the most obvious disadvantages of those cells is their invasive collection procedure, which may cause patients pain and discomfort, while sometimes allowing only for the collection of a low number of cells [157,158].

In this work, an alternative source of MSCs was explored: the Dental Pulp.

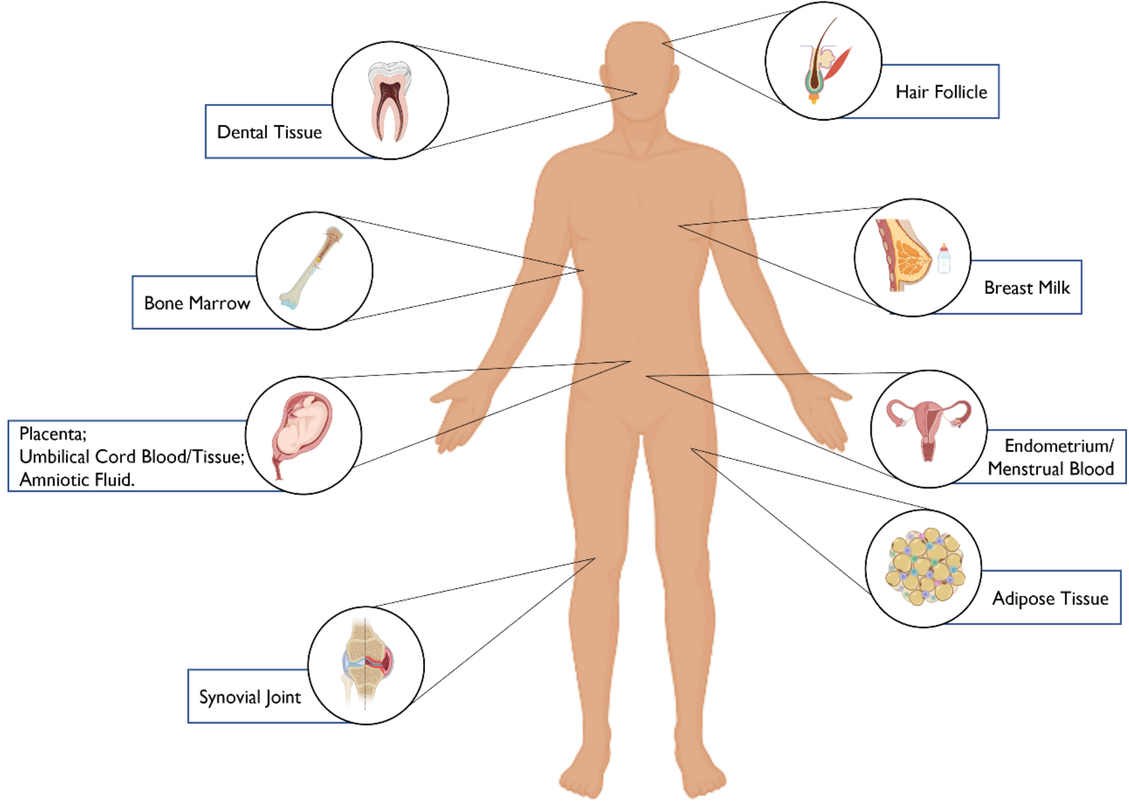


***Figure 6*** *- Different sources of Mesenchymal Stem Cells (MSCs) adapted from Liu et al., 2022* [278]*; Fridman et al., 2018* [158], *Macrin et al., 2017* [279] *(created in biorender.com).*

### *2.3.2. Dental Pulp Stem Cells (DPSCs)*

An interesting study in 2000 [159] introduced to the world a possible new source of stem cells: the dental pulp. The dental pulp is an oral non-mineralized tissue with various cell types, localized in the central pulp cavity and mostly comprises soft tissue with vascular lymphatic elements [160]. Inside it, we may find the so-called Dental Pulp Stem Cells (DPSC). Those

cells have an ectodermal origin derived from neural crest cells [161], more specifically from peripheral nerve-associated glia [162].

In that original study [159], those recently discovered stem cells were compared to BMMSCs, and the evidence they gathered showed that those DPSCs exhibit a higher proliferation rate when compared to Bone Marrow MSCs, while expressing the same pluripotency markers. Thus, this pivotal study became a launching pad for the subsequent exploration of these cells. The impossibility of generating adipocyte cells in the original study was the only lack in classifying DPSCs as MSCs. However, over the following years, more evidence was gathered proving their stem nature. Ultimately, in 2002, the same group that originally assessed their MSCs features was actually able to promote the adipogenic differentiation of those cells using a more specific induction medium. They also confirmed that human DPSC are capable of self- renewal after an *in vivo* transplant [163].

After a few years of constant research, a terminology was established that is still used today, which allows us to distinguish between the different stem cell populations that reside inside the dental pulp (Figure 7). Indeed, depending on the source of the oral cavity from which they are extracted, five different types of dental mesenchymal stem cells (DMSCs) may be distinguished: DPSCs, Stem Cells From Deciduous Teeth (SHEDs) [164], Stem Cells From Apical Papilla (SCAPs) [165], Periodontal Ligament Stem Cells (PDLSCs) [165], and Dental Follicle Stem Cells (DFSCs- precursor cells of PDLSCs [166]).

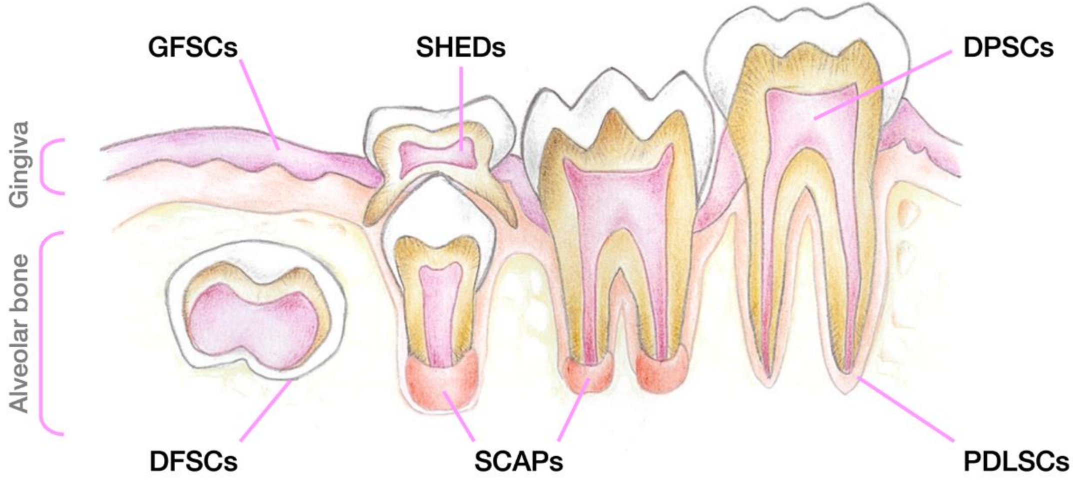


***Figure 7** - Principle sources of Dental Mesenchymal Stem Cells (DMSC) in oral cavity.*

Besides the different oral cavity source, we can distinguish those stem cells by their proliferation rate and potential to differentiation into the several cells. Regarding the

proliferation rate, the Follicle-derived ones seem to have the highest, closely followed by SHEDs, SCAPs, PSLSCs and DPSCs. [167–174]. In Table 3, it is reviewed some experiences done so far, to identify the better cell type for each kind of differentiation.

***Table 3*** *- Osteogenic, Chondrogenic, Adipogenic and Neurogenic Differentiation Potential of the different sources of stem cells from oral cavity*

| Differentiation Potential | | References |
|---|---|---|
| Osteogenic | PDLSCs>DFSCs/SHEDs>DPSCs>SCAPs | [164,168,174–177] |
| Chondrogenic | DPSCs>SCAPs/DFSCs/PDLSCs | [168,176,177] |
| Adipogenic | DFSCs>DPSCs/SCAPs>PDLSCs | [168,177] |
| Neurogenic | SHEDs>PDLSCs>DPSCs>DFSCs>SCAPs | [176,178,179] |

Ever since DMSCs were first identified, a growing number of studies has led to major discoveries in the field. Actually, a report from 2020 [180], distinguishes 3 main "periods" on Dental Mesenchymal Stem Cells (DMSCs) research: (1) discovery and characterization of the different cell populations, a period that goes from 2000 to 2003; (2) mechanistic and preclinical studies, from 2004 to 2012; and, finally (3) *in vivo* characterization and clinical studies, from 2014 to 2019 (present). Some of the most relevant events are described in Figure 8, as well as, the evolution of scientific research in DPSCs field.

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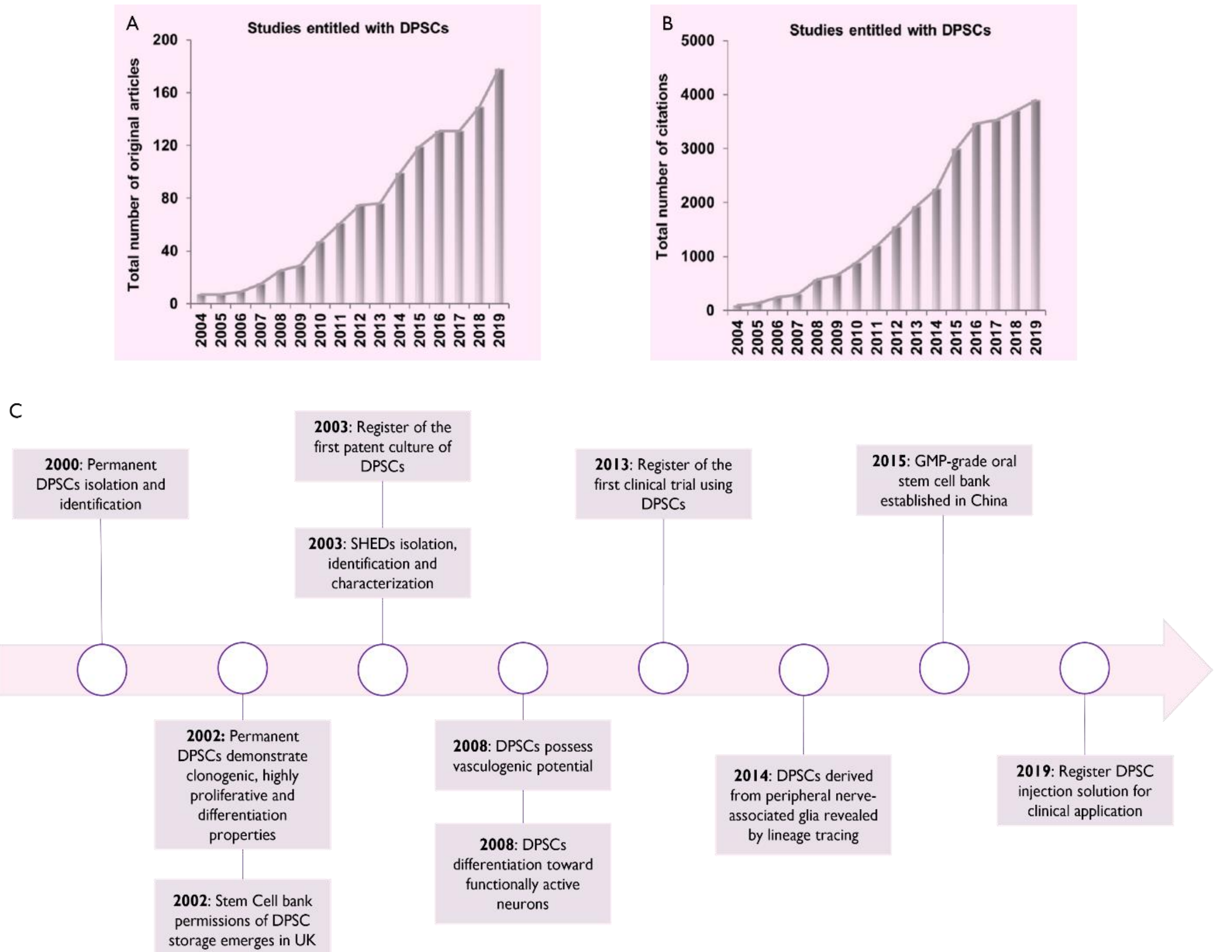



***Figure 8** - Evolution of research in DPSCs field from 2000 until 2019. A: Crescent number of original studies published on DPSCs since their discovery (adapted from Sui et al., 2020* [180]*); B: Increase in citations about DPSCs (adapted from Sui et al., 2020* [180]*). C: Cronological organization of the most relevant discoveries about DPSCs (adapted from Sui et al., 2020* [180]*).*

We are now in 2023, and the existence of these different stem cell populations has now been known for over 20 years. So, DPSC and SHEDs in particular, have been generated from dental pulp for some time. Nevertheless, the majority of the studies involving those cells have focused on their differentiation into chondrocytes for dental repair, with the eventual goal of re-growing teeth from multipotent DMSC cultures [163,181]. Also addressed by a few teams is the potential they hold for stroke therapy. The first study to investigate DPSC in an animal model of stroke dates back to 2009 and used a mechanical extraction method to obtain cells from human third molars. The cells extracted from those teeth were shown to efficiently express the nuclear receptor related 1 protein, which is essential for the dopaminergic system of the brain, and promote, when transplanted, motor functional recovery [182]. After this study was performed, a few others followed, always relying on the use of SC from different dental pulp sources, and being tested *in vivo* in rat models of focal cerebral ischaemia. While it falls completely out of the scope of this review to summarize all those studies, it is worth mentioning that most of them showed really promising results (reviewed in [183]). Curiously,

those cells have been shown to enhance poststroke functional recovery through a non-neural replacement mechanism, i.e., via DPSC-dependent paracrine effects ([184]; reviewed in [183]). And that is probably one of the reasons why this sort of cells have been addressed for their therapeutic potential on many other disorders, affecting various different organs such as kidney (acute renal injury [185,186] and nefritis [187]); lungs (acute lung injury [188]); brain (Parkinson's disease [189,190], Alzheimer's disease [191], cerebral ischemia [192,193]); spinal cord (spinal cord injury [194–197]); liver (liver fibrosis [198–200]); heart (acute myocardial infarction [201,202]); muscle (muscular dystrophy [203–205]); bone (calvarial defect [187,206–208], osteoporosis [209]; skin (wound injury [210,211]); pancreas (diabetes [212,213]) eye (glaucoma [214], cornea trauma [200])and immune system (rheumatoid arthritis [215], autoimmune encephalomyelitis [216] and systemic lupus erythematosus (reviewed in [180,217]).

And if it is true that, for most of these injuries, the evidence gathered so far comes from *in vivo* studies alone, when it comes to the use of DMSCs in oral diseases, the scenario is significantly different, with two clinical studies on pulp regeneration having been launched within the past several years that have achieved breakthroughs in humans (reviewed in [180]). Overall, the results are so good and the possibilities so vast that soon a commercial interest was found in this type of cells. In fact, due to their easy accessibility and favorable therapeutic applications, cell/tissue banking in the dental field are now a reality in several countries, with some of the most well-known ones being BioEDEN (Austin, Texas), Store-a-Tooth (Lexington, Kentucky) Cell Technology (Japan) or the Tooth Bank (Brownsburg, Indiana) (reviewed in [173,218]). And as exciting as these results and perspectives may sound per se, we believe that the overall potential of these stem cells goes far beyond their properties for tissue repair and regeneration. We think, as other authors have also highlighted before, these cells also hold an exceptional potential for neurogenetic disease cell modeling and basic research. In general, DMSCs have a neural crest origin, which makes them a useful source of primary cells for modeling virtually any neurological disorders at the molecular level [219]. Given our interest in LSDs, their monogenic nature and the extremely high prevalence of severe neurological phenotypes in this group of disorders, we considered DMSC as a perfect model to study these disorders.

Interestingly, while their modelling potential has never been addressed for LSDs, as advantageous as it may sound, truth is DPSC are not totally unknown in the field. In fact, back in 2015, Jackson et al. [220] suggested that human MSCs derived from bone marrow and dental pulp could work as an alternative to the use of Hematopoietic Stem Cells (HSCs), in standard

transplantation approaches for the treatment of MPSs. Similarly to what has been discussed in the last section in which we summarized the studies published so far in MPS using iPSCs, in this particular publication, it was the therapeutic potential of the MSCs per se that was analyzed. Actually, none of the MSCs analyzed derived from MPS patients. Instead, all studies were performed in MSCs obtained from healthy donors. This meant that neither the Bone Marrow Mesenchymal Stem Cells (BMMSCs) nor the DPSCs they established had any MPS-related enzymatic defect. Instead, all analyzed cell lines (MSCs and HSCs) were able to produce the different MPS-associated enzymes in the cell layer and secrete low levels of each and every one of them into the surrounding media. However, both MSC types were found to produce significantly higher levels of the majority of MPS enzymes assayed when compared to HSCs, a result that can be considered particularly relevant for therapeutic purposes.

But these authors have done more than just characterizing the normal levels of MPS-related enzymes secreted by the three types of wild-type stem cells, namely Bone Marrow, Dental Pulp and Hematopoietic ones. They also attempted to overexpress, through lentivirus transduction, four different lysosomal enzymes in those same cell lines, to check whether their secretion levels were somewhat similar. Importantly, the evidence they gathered further supported the idea that MSCs (either BMMSC or DPSCs) had higher secretion and production levels of MPS enzymes when compared to HSCs. Also noteworthy, the lentivirus transduction was more efficient in MSCs compared with HSCs.

Then, the authors moved on to investigate *in vitro* the cross correction potential of MPS enzymes secreted from those two different sorts of MSCs in MPS patients' derived fibroblasts, and after confirming the reduction of GAGs accumulation, they also verified that this cross-correction was reached in an M6P-dependent way.

Finally, they also addressed the differentiation ability of the MSCs tested, verifying that both transduced and non-transduced cells maintained that capacity, with only slight differences in the neurogenic process, which appeared to have a slower differentiation pattern in transduced MSCs. As expected, however, MSCs derived from dental pulp had a premature upregulation on mature neuron markers, when compared with those derived from bone marrow.

Altogether, these results provided the *in vitro* proof of principle on the therapeutic potential of DPSCs and Bone Marrow MSCs as an isolated therapy or even combined therapy with the standard HSCTs. To the best of our knowledge, no follow-up studies or in vivo assessments have yet been published on this subject, even though its overall results seem extremely promising.

To the best of our knowledge, MPS patient-derived DPSCs had never been used for differentiation into specific cell types even though they represent a natural source of stem cells that may be used to investigate human disease especially for the infantile forms of these disorders. In fact, taking into account that the most severe forms of MPSs are pediatric, there is one particular population of stem cells in the dental pulp that seems particularly suitable to study them: SHEDs. Among their numerous advantages, which include a high proliferation rate and the greater tendency to generate both skeletal and brain cells, SHEDs collection does not require the active removal of teeth, only their natural fall, and this is certainly an advantage for children who may already be dealing with undue stress and pain.

In general, the higher the number of genotypes we collect the larger the spectrum of future applications our DPSC-derived LSD neuronal cultures may have not only in our lab but also for other researchers in the field. In addition, with the advances of new gene editing technologies, such as CRISPR/Cas base editing, prime editing and the "older" transcription activator-like effector nucleases (TALEN) and zinc finger nucleases (ZFN), arised the possibility to generate pairs of isogenic lines that facilitate the study of the function of a given gene and the role that different mutations play in the pathophysiological mechanisms of the respective diseases. This approach has been increasingly applied to iPSC lines and could also be very useful in the case of our DPSC-derived cell lines.

Still another naturally-occurring source of stem cells are human urine-derived stem cells (USCs), a type of MSCs with proliferation and multi-potent differentiation potential that can be readily obtained from voided urine using an non-invasive protocol and with minimum ethical restriction. These cells express surface markers of MSCs, but not of hematopoietic stem cells, express the stemness-related genes *NANOG* and *Oct3-4* and show telomerase activity, not forming teratomas *in vivo* after being subcutaneously implanted in nude mice [221–224]. When cultured in appropriate media, USCs may differentiate into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic, and neurogenic lineages. Interestingly, USCs may be established from individuals of any age, despite Gao et al. have shown that those isolated from children (5 to 14 year-old) have higher proliferation, lower tendency to senescence, and stronger osteogenic capacity than those from middle-aged (30 to 40 years-old) and elder (65 to 75 year-old) individuals [223]. This property allows to significantly expand the cohort of patients accessible to be studied. Overall, USCs are yet another alternative source of SCs that can be used as a valuable *in vitro* model to study genetic diseases, with potential applications in regenerative medicine, cell therapy, diagnostic testing and drug screening [225].

# Materials and Methods

# Materials and Methods

## 1. Primary cell culture of Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) *in house*

Canines and incisors baby teeth were obtained from children aged 8 to 12, right after falling, from both controls (with no associated disease) and patients (with MPS II and MPS VI), who voluntarily donated them to the project.

Overall, the protocol for the collection, transport and isolation of SHEDs, as well as that for their subsequent passage, freezing and thawing, was adapted from an original proceeding published in 2017 by Goorha and Reiter, on *Current Protocols in Human Genetics* [226].

### 1.1. Collection, Transport and isolation of control- and MPS-derived SHEDs

Two independent call for volunteer approaches were followed, depending on whether control or diseased samples were being requested. Both approaches were publicized under the title "The 2020s Tooth Fairy Project".

In brief:

1. To apply for healthy volunteers, whose derived SHEDs would then serve as controls for subsequent studies, an informal, yet extremely successful call for volunteers was carried out recurring mostly to social media and science communication blogs. Basically, those platforms were used to reach of the non-scientific community to and briefly explain why baby teeth were necessary for this particular research project (see *Annex* 2).

2. To identify MPS patients, whose families would be willing to donate a recently fallen deciduous tooth from their affected children, several pediatricians from the major Portuguese Reference centers for Metabolic Diseases (namely, LSDs) were approached, namely: Elisa Leão Teles (from *Centro Hospitalar Universitário de São João*, CHUSJ); Esmeralda Martins (from *Centro Hospitalar Universitário do Porto*, CHUP); Luísa Diogo and Paula Garcia Matos (from *Centro Hospitalar Universitário de Coimbra*, CHUC) and Patrícia Janeiro (from *Centro Hospitalar Universitário* Lisboa Norte, CHULN).

   Additionally, the major Portuguese Patient Associations in the field (namely Sanfilippo Portugal, Associação Portuguesa de Doenças do Lisossoma, APL and RD- Portugal) were also enrolled, having actively contributed to spread the news among their associates, and enlightening the families on the study itself (see Annex 2).

### *1.1.1. Preparation of "tooth kits" to be sent to the families*

While the overall protocol here described allows for the collection of exfoliated teeth from remote locations and their transport to the laboratory at room temperature, to conserve the baby teeth and avoid possible contamination during this whole process, it is necessary to keep them in an appropriate medium, which will be, from now on, designated *Transport Medium* (see *Annex* 3 - *Transport Medium*). In fact, the *Transport Medium* will not only allow for the teeth roots to remain moist during transportation, but also actively contribute to decrease contaminations, as it contains anti-bacterial and anti-fungal reagents.

As soon as a subject or family volunteered to join this study, a “tooth collection kit” was prepared to be sent to the family, which included a parafilm-sealed Falcon tube filled with adequate *Transport Medium* accompanied by return instructions, a biohazard bag, plus a pre-filled delivery form.

Also included in the kit was an Informed Consent Form to be filled by the participant’s legal representative (see *Annex* 2), a summary of the project and its objectives (*Annex* 2) and a flyer with major recommendations and frequently asked questions (*Annex* 2).

The families were instructed to store the Falcon tube in the refrigerator (4°C) and to place the tooth in it, right after its fall and sent to the laboratory within 24 hours. In the informative material sent along with the kit, emphasis was given to the fact that the cells that reside inside the tooth are only available for a couple of days, being crucial to do the procedure as soon as possible.

All kits were sent to their respective families by regular mail, at room temperature, in adequate padded envelopes.

### *1.1.2. Dental pulp extraction and establishment of the primary SHED cultures*

DPSCs and SHEDs reside inside the dental pulp tissue. Therefore, one has to break the teeth open and extract the dental pulp to assess those cells. Still, DPSC and SHED cells’ isolation does not rely exclusively on a mechanical process: it also requires a biochemical digestion of the pulp tissues. Over the following paragraphs, the method to ensure their successful isolation is carefully described.

To assure a controlled and sterile environment, every step of this procedure was executed in a laminar flow chamber (biological safety cabinet class II). Furthermore, whenever a tooth was received in the laboratory, its *Transport Medium* was carefully inspected for contaminants

and if it appeared cloudy, the sample was immediately discarded. In fact, this protocol was only carried out when the *Transport Medium* showed no signs of contamination.

First, the *Transport Medium* was aspirated and discarded. The tooth was then broken, preferably all at once with a single blow, with the help of a sterilized hammer wrapped in parafilm. Subsequently, using sterile tweezers and a scalpel blade, pieces of pulp were extracted and cut into smaller pieces. Those pieces were transferred to a 15 mL Falcon tube and centrifuged for 5 minutes at 2000 rpm. The resulting pellet was then resuspended with an appropriate medium (see *Annex* 3 - *DPSC Culture Medium*), pre-warmed (37ºC) and supplemented with 1-4 mg/mL *Dispase II* (Neutral Protease Grade II, Roche, Basel, Switzerland) and 3 mg/mL *Collagenase* (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and incubated for 1 hour at 37ºC.

Following enzymatic digestion, the Falcon tube was centrifuged once again under the same conditions, and the resulting supernatant aspirated. The remaining pellet was resuspended in 1 mL of *DPSC Culture Medium*. The dental pulp-derived cells were then seeded in a cell culture 12-well plate previously coated with either *poly-D-lysine* (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) or *vitronectin* (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and moved to an incubator at 37ºC and 5% $CO_2$, for 24 hours.

On the following day, the medium from that original well was removed and centrifugated on a 1,5 mL Eppendorf, the supernatant was discarded, and the pellet resuspended in 1 mL of *DPSC Culture Medium*. The whole content was then transferred to another similarly coated plate well and left to incubate again for at least 2 or 3 days, at 37ºC, 5% $CO_2$.

Thereafter, the culture was maintained under conditions, which were not particularly different from those used for any other patient-derived adherent cell culture (e.g. fibroblasts), with daily morphological observation and medium changes at least every other day.

## 1.2. Culture and Maintenance of the established SHED cell lines

When the cells first reached 80-90% confluence, they were ready for subsequent passage and storage. Later on, higher passages were also pelleted for subsequent analyses.

Over the next paragraphs, there is a brief summary of all those protocols.

### *1.2.1. Storage and passage of cell cultures*

To suspend the adherent cells, *Accutase* (GRiSP, Porto, Portugal) was applied for 5 minutes at room temperature. Then, *DPSC Culture Medium* was added (twice of the *Accutase* volume).

From the total volume, 2/3 were stored (for short and long-term use) and the remaining was platted in another 6-well plate previously coated with *poly-D-lysine* or *vitronectin*.

Cells were stored in a cryotube with *DPSC Culture Medium* supplemented with Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, Missouri, USA), which was either kept at - 80°C for short term storage, on in liquid nitrogen, for long-term storage. Either way, cryopreserved SHEDs will be ready for subsequent uses.

#### *1.2.2. Generation of pellets from the different established SHED cell cultures*

To perform the characterization of the cell lines, it is usually necessary to generate pellets from those cells. The procedure starts by applying accutase during 5 minutes to take off the cells from the plate well. In the case of a 6-well plate, the *Accutase* quantity is usually 500 µL. After that, a double amount of *DPSC Culture Medium* (500*2=1000 µL) is added, followed by centrifugation for 5 minutes at 13.000 rpm. Then, to wash any traces of the *DPSC Culture Medium*, the pellet was resuspended in *PBS 1X*, followed by another centrifugation. The supernatant was aspirated, and the pellets were kept at - 80°C until posterior use.

## 2. Confirmation of the stemness potential of the established SHED cell lines and validation of their MSC identity

There are several requirements a cell has to fulfil to be classified as part of a MSC population, according with the International Society for Cell & Gene Therapy (ISCT)'s recommendations.

Different methods were employed to characterize the established cell lines and validate their identity, namely quantitative gene expression analysis of human MSCs and pluripotency markers by qRT-PCR, plus the verification of the osteogenic, chondrogenic, and adipogenic differentiation. Details on both approaches are given throughout the following sections.

### 2.1. Assessment of the Mesenchymal Stem Cell identity of the established SHED cell lines

#### *2.1.1. Total RNA extraction*

To extract the total RNA from the stored SHED pellets, the *GRS Total RNA – Blood & Cultured Cells* kit (GRiSP, Porto, Portugal) was used, following the manufacturer's instructions. The reagents used in this protocol were all provided by the kit, except for *β-mercaptoethanol* and 70% ethanol.

Briefly, this protocol involves several steps that start with cell lysis. The cellular pellet was resuspended in 100 µL of *Red Blood Cell Lysis Buffer* and lysed by shaking vigorously with 400 µL of *Buffer R1* and 4 µL of *β-mercaptoethanol*, incubating at room temperature for 5 minutes. Then, 500 µL of 70% ethanol were added to the lysate and 500 µL of this sample mixture transferred to a *RNA mini spin column* followed by centrifugation at 14.000g-16.000g for 1 minute. After discarding the flow-through, this step was repeated for the remaining sample. This step was followed by a wash step with the *Wash Buffer 2*, which allows the RNA to bind to the column. Then, to eliminate any DNA residues that could be present in the sample, a mixture of 45 µL of *DNase I reaction buffer* and 5 µL of *DNase I solution* was also added and the resulting solution incubated for 10-15 minutes at room temperature. Several washes were then performed, always with centrifugations in between and throwing away the flow-through. After that, a longer centrifugation (3 minutes) was done to dry the column matrix. Finally, to promote elution of the sample, 50 µL of *RNase-free water* were applied to the center of the column and incubated for 1-2 minutes at room temperature. To improve the yield, a subsequent 1minute-centrifugation was performed. The concentration of RNA was then quantified with the UV-Vis spectrophotometer *NanoDrop® ND-1000* (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and stored at -80°C when not used immediately.

#### *2.1.2. cDNA synthesis*

After RNA extraction and quantification, the synthesis of the first-strand cDNA was performed with the *Ready-To-Go™ You-Prime First-Strand Beads* (Cytiva, Marlborough, Massachusetts, USA), according to the manufacturer's instructions.

Taking into account the previous RNA quantification results, a volume correspondent to 0.5-1 µg of RNA was placed in a 1.5 mL Eppendorf tube, which was then filled with *RNase-free water* to a total volume of 30 µL. Then, each reaction was performed according to this simple protocol: the RNA solution was incubated at 65°C for 10 minutes to dismantle the RNA secondary structures, followed by a thermal shock on ice for 2 minutes. That solution was then transferred to one of the thin-walled 0.5 mL tubes containing the pre-formulated single-dose reaction beads, which are included in the kit. According to the manufacturer, each bead contains dNTPs, murine reverse transcriptase, RNAguard™, and *RNase/DNase-free BSA*. Next, 2 µL of *RNase-free water* and 1 µL *oligo(dT)18 primer mix (50 µM)* (NZYTech, Lisboa, Portugal) were added to the tubes containing the RNA and the beads, bringing up the solution to a total volume of 33 µL.

Then, the tubes were left to incubate at room temperature for 1 minute, briefly vortexed and, finally, incubated at 37°C for 60 minutes to allow the cDNA synthesis. The cDNA products were stored at -20°C.

#### *2.1.3. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)*

To confirm the DPSC/MSC phenotype identity of the established cell lines, quantitative Real-Time polymerase chain reaction (qRT-PCR) was performed for the following MSCs' related genes (primers from Bio-Rad Laboratories, Hercules, California, USA): *CD34* (qHsaCID0007456), *CD90* (qHsaCED0036661), *CD73* (qHsaCID0036556), *CD105* (qHsaCID0010800), *SOX2* (qHsaCED0036871), *OCT3-4* (qHsaCED0038334), and *MHC Class II/HLA-DRA* (qHsaCED0037296). The following housekeeping genes were also used: β-*actin* (qHsaCED0036269) and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) (qHsaCED0038674).

qPCR was performed in a CFX96 Touch Deep Well (Bio-Rad Laboratories, Hercules, California, USA) apparatus using the *SsoAdvanced Universal SYBR® Green Supermix* (Bio-Rad Laboratories, Hercules, California, USA). All plates were designed to contain duplicates of targeted human genes as well as a negative control. Recommended PrimePCR cycling protocol was employed in all cases: 95°C for 2 min (activation), 40 cycles comprising 95°C for 5 s (denaturation), 60°C for 30 s (annealing), and 65–95°C (0.5°C increments), 5 s/step (melt curve). Data was processed using BioRad CFX® Manager Software 3.1 (Bio Rad Laboratories, Hercules, California, USA). For each well, the value of the cycle threshold (*Ct*) was assessed. Fold differences were calculated using the standard ΔCq method with *GAPDH* and β-*actin* as housekeeping genes.

### 2.2. Endodermal, Mesodermal, and Ectodermal Differentiation of SHEDs-derived cell lines

One of the requirements a certain cell line has to fulfil to be classified as MSC is the ability to differentiate into three different cell types: adipocytes, chondrocytes and osteocytes. Therefore, apart from the qRT-PCR analysis described in 2.1, performed to confirm their MSC phenotype, the actual capacity of the established SHED cell lines had to differentiate into those cell types was also assessed by incubating them with specific differentiation media, and carefully monitoring the changes it caused to their original fibroblast-like morphology.

Additionally, a fourth protocol was also performed, to promote the differentiation of the established SHED cell lines into mixed neuronal and glial cultures. One such protocol, not only allowed for the assessment of whether those cells were able to differentiate into cells from

another germ layer (namely, ectoderm), but also to evaluate the possibility to generate a disease-relevant neurological model, for the pathologies under analysis.

The different protocols used are briefly summarized in the following sections.

#### *2.2.1. Adipogenic Differentiation*

To differentiate the SHED cells into adipocytes, the *StemPro® Adipogenesis Differentiation Kit* (Gibco®, Life Technologies, Carlsbad, California, USA) was used. Before the adipogenesis differentiation medium was first applied, cells were maintained for 2 passages in the standard *DPSC Culture Medium* until they reached the 60-80% confluence. As soon as each cell line reached that optimal confluence, the adipogenesis medium was added. From then on, medium was changed every 4 days, and pellets were made at 7, 14, and 21 days, to allow for additional analysis, *a posteriori*.

#### *2.2.2. Chondrogenic Differentiation*

To differentiate the established SHED cell lines into chondrocytes, another differentiation kit from the same company was used: the *StemPro® Chondrogenesis Differentiation Kit* (Gibco®, Life Technologies, Carlsbad, California, USA). Again, before applying the chondrogenesis differentiation medium, cells were maintained for 2 passages in the standard *DPSC Culture Medium*. As soon as they reached 60-80% confluence, the chondrogenesis medium was added. From then on, medium was changed every 3 days. Pellets were made after 14 and 21 days of incubation, according with the recommendations from the literature.

#### *2.2.3. Osteogenic Differentiation*

To differentiate the SHED cells into osteocytes, the *StemPro® Osteogenesis Differentiation Kit* (Gibco®, Life Technologies, Carlsbad, California, USA) was used. As already referred for the other differentiation kits, before the osteogenesis differentiation protocol was started, cells were maintained for 2 passages in the standard *DPSC Culture Medium* until they reached the 60-80% confluence. As soon as each cell line reached that optimal confluence, the osteogenesis medium was added. From then on, medium was changed every 4 days, and pellets were made at two different time points: day 14 and day 21.

#### *2.2.4. Neurogenic Differentiation*

To promote SHED cells differentiation into neural cells, a different kit was used: the *Human ES/iPS Neurogenesis Kit* (Milipore®, Burlington, Massachusetts, USA).

The kit protocol was specifically designed for neuronal differentiation from iPSCs, but has also been validated in other stem cells. Like most neuronal differentiation protocols, it involves 3 steps: Epigenetic Reprogramming, Neural Differentiation, and Neural Maturation [226].

In the case of the *Human ES/iPS Neurogenesis Kit* those 3 steps translate into four different media, each one with a specific composition: two independent *Neural Induction Media* (*NIM1* and *NIM2*), one *Neural Expansion Medium* designated *ENStem-A*, and, finally, one *Neuronal Differentiation Medium* (*NDM*).

Briefly, the protocol may be summarized as follows: *NIM1* was applied for 5 days, and *NIM2* in the 5 following days. Thus, the whole induction stage took 10 days in total. Stage 2, Neural Expansion, relied on the use of the *ENStem-A* medium, which was applied to the cells for several weeks until cells could be passaged and stored (cryopreserved in freezing medium). Finally, stage 3 lasted 10 to 25 additional days, in which the cells were incubated with the Neuronal Differentiation Medium (*NDM*). In all stages of this protocol, the medium was changed every other day.

For every attempted differentiation protocol, cells viability and morphology were checked using an inverted light microscope and any relevant alternation noted.

## 2.3. Neuronal Markers assessment in the established SHED cell lines by immunocytochemistry

While not initially envisaged, an additional characterization protocol was also performed: an immunocytochemistry assay with neuronal markers, which was performed to confirm the DMSC early commitment to their so-called "neuronal fate". This study was performed in primary SHED cells, before any neuronal differentiation protocol was attempted, and relied on the use of a commercially available kit, whose protocol is briefly summarized in the next section.

### *2.3.1. Immunocytochemistry assay*

The commercial kit used for this assessment was the *Human Neural Stem Cell Immunocytochemistry Kit* (Molecular Probes®, Eugene, Oregon, USA), which stained Nestin, PAX6, SOX1, and SOX2. The assay was performed following the manufacturer's instructions.

Cells were seeded in Lab-TekII chamber slide plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and left to reach an adequate confluence (≈60-70%). To ensure that the cells would not detach during subsequent steps, chambers were filled with 100 µL of *Fixative solution* (provided by the kit) and left to incubate at room temperature for 15 minutes. After

that period, the fixative solution was removed. At this point, the samples could be stored at 4ºC in *Wash Buffer* (provided by the kit, and diluted to 1X with water), or used immediately.

After that initial fixation step, the procedure followed outside the laminar flow, with a subsequent incubation with *Permeabilization solution* at room temperature. After 15 minutes, the *Permeabilization solution* was removed and *Blocking solution* was applied for 1 hour, still at room temperature.

The primary antibodies (*anti-mouse-NESTIN*, *anti-goat-SOX1, anti-rabbit-PAX6*, and *anti-rabbit-SOX2*) diluted to 1X with *Blocking Solution*, were then applied and incubated (100 µL) overnight at 4ºC. In the next day, those solutions were removed and 3 wash steps were performed. Finally, the secondary antibodies (*Alexa Fluor® 488 donkey anti-mouse*; *Alexa Fluor® 488 donkey anti-goat*; *Alexa Fluor® 555 donkey anti-rabbit*) were also diluted to 1X with *Blocking Solution* and applied to their respective chamber wells, incubated for 1 hour at room temperature followed by 3 wash steps.

Finally, to allow for the subsequent visualization of staining, the wash buffer was aspirated, the chamber dismantled and 2 drops of *NucBlue® Fixed Cell Solution (DAPI)* applied to the slide, which was left to incubate for at least 5 minutes. The images were acquired by Fluorescence Microscopy (*Automated UpRight Microscope System Leica DM 4000B; Leica Application Suite v.3.7.0*).

## 3. Assessment of the LSD-associated subcellular phenotype(s) in the established MPS patient-derived SHEDs

To confirm that the established SHED cell lines were able to present the primary defect underlying the MPS phenotype in the patients from whom they were derived, a careful molecular characterization of their associated genotypes was performed, together with a quantification of each one's defective enzyme. Additional assessments were made to understand whether these cell lines were able to recapitulate other LSD-associated subcellular phenotypes, such as the presence of storage material and the abnormal distribution and/or quantity of lysosomes.

### 3.1. Molecular confirmation of the disease-causing enzymatic defect(s) in each established cell line

The molecular characterization of disease cell lines was performed through amplification and sequencing of the genes, which were known to be defective in each case: *IDS* gene for the MPS II cell lines, and *ARSB* gene for the MPS VI cell line.

### 3.1.1. *Molecular characterization by gDNA analysis*

#### *3.1.1.1.gDNA extraction*

gDNA was automatically extracted from the stored pellets, using the *EZ1 DNA tissue extraction kit* (Qiagen, Hilden, Germany). Briefly, cell pellets were lysed with a mixture of 190 µL of *Lysis Buffer* and 10 µL of *Proteinase K*, incubated at 56°C. As soon as the pellet was completely dissolved, 200 µL of those cell lysates were transferred to appropriate 2 mL tubes and placed in the biorobot, together with the necessary tips and tip-holders, which warrant the subsequent washes that culminate in a final elution of the gDNA sample in a previously defined volume (usually 50 µL).

#### 3.1.1.2. *PCR amplification of the target MPS genes*

After extraction, genomic DNA was used for the amplification of the *IDS* and *ARSB* genes (all exons and their surrounding intronic regions) using previously reported primers [227,228]. Each PCR reaction was carried out using approximately 40 ng of genomic DNA, 1X the PCR reaction mix *ImmoMix™ Red* (Bioline, London, UK) and 0.5 µM of each primer. For some particular fragments, *Betaine* (Sigma-Aldrich, St. Louis, Missouri, USA) and/or *DMSO* were also used to enhance the PCR amplification of the target region (see Table 4 for further details). The amplification program was composed of an initial denaturation step at 95°C for 7 min, followed by 30 cycles of denaturation, annealing and extension according to the conditions highlighted in Table 5. The final extension was completed by 5 min at 72°C.

***Table 4*** *-Primer sequence of gIDS and gARSB and respective annealing conditions. *added 0.5 µL DMSO and 0.5 µL Betaine to the reaction.*

| | Exon | Primers Designation | Sequence (5'→ 3') | $T_{annealing}$(°C) |
|---|---|---|---|---|
| Disease: MPSII Gene: *IDS* | 1 | *gIDS* 1F | GCAAAAAGACGGGTAACTGC | 56 |
| | | *gIDS* 1R | AGGGAGGAAGGGAGAAGAGA | |
| | 2+3 | *gIDS* 2+3F | TCCAGCCTTGGGCCTCTTAG | 58 |
| | | *gIDS* 2+3R | AGAGAACCCAGACTCTGGACA | |
| | 4 | *gIDS* 4F | GTTCCACTTGCCCATTTGTT | 58 |
| | | *gIDS* 4R | ACCAGCTTCACAGAACATGC | |
| | 5 | *gIDS* 5F | CGTGAAGGGCTGATTATGTG | 58 |
| | | *gIDS* 5R | ATGTAGCCACCTTCCCTGTG | |
| | 6 | *gIDS* 6F | ACGTGGGAATGCTAGTGAG | 58 |
| | | *gIDS* 6R | GGTGGAGTTGTGTCTACTGAGAA | |
| | 7 | *gIDS* 7F | GATTGGGAGAGATGCACAGG | 62 |
| | | *gIDS* 7R | CCACTGGTTCACAAAAGAGAA | |
| | 8 | *gIDS* 8F | ACAAGCTGTGGTATGATGAT | 58 |
| | | *gIDS* 8R | TAAAGGTGATCTTACTGTCAA | |
| | 9 | *gIDS* 9F | AGGTGGTGTTTCTAAACGTCTG | 62 |
| | | *gIDS* 9R | CAAAACGACCAGCTCTAACTC | |
| | JP | *gIDS* P1F | TGGGCATCTCTGATGGGC | 58 |
| | | *gIDS* P1R | AACAGTGAGCTGTGGAACTGCA | |

| | | | | |
|---|---|---|---|---|
| | JD | *gIDS* D1F | CTCTCCCTGAGCTCATCATTC | 58 |
| | | *gIDS* D1R | AACAGTGAGCTGTGGAACTGCA | |
| Disease: MPSVI Gene: ARSB | P | *gARSB* PF | CTGTTTGCTAGTGGGGAGGA | 60 |
| | | *gARSB* PR | CCCCTTGTACCGCTGATAGA | |
| | 1 | *gARSB* 1F | GTTCGTCTCTGGCTCCTCCT | 58* |
| | | *gARSB* 1R | GCCTGGAAGAGCGAGGTT | |
| | 2 | *gARSB* 2F | GAAGGCCATTTTATCTGCTTG | 60 |
| | | *gARSB* 2R | AAAGCAGCCCCATTACAGTG | |
| | 3 | *gARSB* 3F | TAGCCTCGTCACGGGTAATC | 60 |
| | | *gARSB* 3R | CAACAATGGCCTTTTCCTACA | |
| | 4 | *gARSB* 4F | TGCATTCTGTAGGTTGTCTTGA | 60 |
| | | *gARSB* 4R | TCCACAATTACCATGTCTCCA | |
| | 5 | *gARSB* 5F | GGGAAAAGGCAAGGAATTTT | 60 |
| | | *gARSB* 5R | TCATGTATTTGTAAGCTGAACTATCA | |
| | 6 | *gARSB* 6F | TTCAAAGGGTCCCAGAATCA | 60 |
| | | *gARSB* 6R | AGCACACTGCCCTCTGAGAT | |
| | 7 | *gARSB* 7F | TTGCGGTGGTTTATGACTGA | 60 |
| | | *gARSB* 7R | GGTGGGAAACGGTTAGAACA | |
| | 8 | *gARSB* 8F | CCACACCCACAACCCAGT | 60 |
| | | *gARSB* 8R | CCTCGGTGTGGTTTAAGAGC | |

***Table 5*** *- Conditions of PCR amplification. *Annealing temperature may differ depending on each fragment analyzed (see **Table 4**).*

| Steps | Temperature (°C) | Time (Min:Sec) | Cycles |
|---|---|---|---|
| Denaturation | 95.0 | 07:00 | x1 |
| | 94.0 | 00:45 | x30 |
| Annealing | * | 00:45 | |
| Extension | 72.0 | 02:00 | |
| | 72.0 | 10:00 | x1 |
| | 4.0 | Pause | |

*3.1.1.3. Analysis of the PCR reaction by agarose gel electrophoresis*

The amplification through PCR reaction was verified by electrophoresis in 2% agarose gel, immersed in 1X *Tris-Acetate-EDTA buffer* (1X TAE), and stained with 7 µL of *ethidium bromide* (Sigma-Aldrich, St. Louis, Missouri, USA). Each gel well was filled with 5 µL of PCR product and one well with 7 µL of 100 bp molecular weight marker (*DNA Ladder ready-to-load*; Bioron, Romerberg, Germany). Then, an electric current with 110V for 30-45 minutes was applied.

The gel was visualized under UV light and the Molecular Imager® Gel Doc XR+/*Image Lab*™ (Bio-Rad, Hercules, California, USA) was used for image acquisition.

#### 3.1.1.4. *Purification of PCR product*

The generated PCR products were purified with Illustra ExoStar 1-Step™ (GE Healthcare, Buckinghamshire, UK) or after gel extraction with *Wizard® SV Gel and PCR clean-up system* (Promega, Madison, USA).

*Illustra ExoStar 1-Step*™ purification was performed whenever only one band, of the expected size, was observed in the agarose gel, as a result of a single PCR reaction. In that case, 3 µL of the amplified a PCR tube were mixed with 1 µL of *ExoStar 1-Step kit*, an adaptation of the original manufacturer's protocol that allows for a higher number of purification reactions per kit. Then, the preparation underwent the following incubation protocol: 37°C by 30 minutes followed by 15 minutes at 80°C.

When more than one band corresponding to the same PCR product was seen in the agarose gel, the individual bands were cut and purified with the *Wizard® SV Gel and PCR Clean-Up System Kit* (Promega, Madison, USA), according with the manufacturer's instructions. Briefly, the bands of interest were excised from the agarose gel and dissolved in the membrane *Binding Solution* provided in the kit, at 50-65°C according to a proportion of 10 µL of solution per 10 mg of gel slice. The dissolved gel mixture was then added to a microcolumn assembly and left to incubate at room temperature for 1 minute. Right after that incubation, the assembled column was centrifuged and the flowthrough discarded. Then, a series of standardized washes, centrifugations and wash-through removals was performed to remove all possible contaminants. Finally, DNA was eluted by adding 50 µL of nuclease-free water to the microcolumn and collected to a 1.5 mL Eppendorf tube.

#### 3.1.1.5. *Sequencing of the fragments obtained*

After purification, the PCR products underwent a sequencing reaction under the conditions described in Table 6. For each purified PCR amplicon, two separate sequencing reactions were performed, one with the forward primer and the other with the reverse primer. Both reactions were composed of 1 µL of *BigDye®*, 1 µL of *BigDye® buffer (components of the BigDye® Terminator v1.1 Cycle Sequencing Kit* (Applied Biosystems, Foster City, California, USA), 0.5 µL of *primer at* 5µM, 2 µL of the amplified product, and 5.5 µL of sterile water to complete a final volume of 10 µL.

*Table 6 - Sequencing Conditions of PCR products obtained from gDNA fragments.*

| Steps | Temperature (°C) | Time (Min:Sec) | Cycles |
|---|---|---|---|
| 1 | 96.0 | 10:00 | x1 |
| 2 | 96.0 | 00:10 | x25 |
| 3 | 50.0 | 00:05 | |
| 4 | 60.0 | 04:00 | |
| 5 | 4.0 | Pause | x1 |

The sequencing products were then purified and separated through capillary electrophoresis in an *ABI Prism 3130 Genetic Analized* (Applied Biosystems, Foster City, California, USA). The resulting electropherograms were analyzed using the *Finch TV software* (Geospiza, Seattle, USA) and compared with the reference sequences of the target genes, which are available in the *Ensembl database* (*IDS*: ENST00000340855.11; *ARSB*: ENST00000264914.10) with the help of the *Clustal Omega bioinformatic* tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

### *3.1.2. Confirmatory molecular studies by cDNA analysis*

The presence of the variants detected in the genomic DNA samples of all patients here described was also confirmed at cDNA level. Briefly, total RNA was extracted and reverse transcribed as described in sections 2.1.1 and 2.1.2, respectively.

#### *3.1.2.1. Amplification of the* IDS *and* ARSB *transcripts*

To confirm the presence of the disease-causing mutations in the cDNA samples and further analyze the presence of any alternative transcripts, cDNA amplification was performed. cDNA amplifications were performed in a total volume of 25 µL using the Hot-Start PCR mastermix *ImmoMix*™ *Red* (Bioline, London, UK) with primers at 0.5 µM and 5 µL of cDNA. Again, for a few particular fragments, *Betaine*, *DMSO*, or a combination of both were used (see Table 7 for details).

The amplification reactions were performed in the thermocyclers as referred above under the conditions described in (see section 3.1.1.2). Primer sequences used to amplify *IDS* and *ARSB* cDNA sequences are listed bellow, in Table 7, along with their respective annealing temperatures and any specific amplification requirements.

***Table 7*** *- Primer sequence of cIDS and cARSB and respective annealing conditions. *added 0.5 µL DMSO and 0.5 µL Betaine to the reaction.*

| | Fragment | Primers Designation | Sequence (5'→ 3') | $T_{annealing(°C)}$ |
|---|---|---|---|---|
| Disease: MPSII<br>Gene: *IDS* | 1 | *cIDS* 1F | CTGTGTTGCGCAGTCTTCAT | 60 |
| | | *cIDS* 1R | GGGGTATCTGAAGGGGATGT | |
| | 2 | *cIDS* 2F | CTGTGGATGTGCTGGATGTT | 58 |
| | | *cIDS* 2R | GGGTCGAGGTAAGGGAAAAG | |
| | 3 | *cIDS* 3F | GATGTTGCTACCCATGTTCC | 58* |
| | | *cIDS* 3R | CAAAACGACCAGCTCTAACTC | |
| Disease: MPSVI<br>Gene: *ARSB* | 1 | *cARSB* 1F | GCAGCCCAGTTCCTCATTCT | 56 |
| | | *cARSB* 1R | GGCAGGAGTTTTTCATCCAG | |
| | 2 | *cARSB* 2F | CTGCTCACTGGCCGCTA | 60 |
| | | *cARSB* 2R | GTGTTGTTCCAGAGCCCACT | |
| | 3 | *cARSB* 3F | TCTCCAGTCTGTGCATGAGC | 60 |
| | | *cARSB* 3R | GTGGAGGGAACCAGTAACCA | |
| | 4 | *cARSB* 4F | GCTCCAGCAAAGGATGACTC | 60 |
| | | *cARSB* 4R | GGTTTTCTAGCCTCCCTGAAA | |

The analysis of the RT-PCR reaction by agarose gel electrophoresis, subsequent PCR products purification and sequencing were all performed following the exact same protocols described for gDNA analyses in sections 3.1.1.3., 3.1.1.4, and 3.1.1.5.

### 3.2. Biochemical confirmation of the disease-causing enzymatic defect in each established cell line by the measurement of enzymatic activities

The molecular defects detected by Sanger sequencing were further validated biochemically, by enzyme activity quantification. Different methods and several lysosomal enzymes were assessed, according to the methods and rationale described bellow.

#### *3.2.1. Quantitation of total protein in SHED cell pellets*

Cell homogenates were prepared by sonication of cell pellets in water. Then their protein concentration was determined using the *Pierce™ BCA Protein Assay Kit* (TermoFisher Scientific, Waltham, Massachusetts, USA) and measured by spectrophotometer (VICTOR® Nivo™ Plate

Reader, PerkinElmer Inc, Waltham, Massachusetts, USA), according to the manufacturer's instructions.

This kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. It has been shown to detect total protein concentrations from 20 to 2.000 µg/mL using a simple two-component system: *Reagent A*, a carbonate buffer containing BCA reagent, and *Reagent B*, a cupric sulfate solution, which are combined to make an apple green–colored working solution that turns purple after 30 minutes at 37°C in the presence of protein. Protein concentrations are then determined using as reference, standards of *bovine serum albumin* (BSA) with known concentration. In order to allow that accurate protein concentration determination, a series of dilutions of known BSA concentrations have to be prepared alongside the unknown samples and the concentration of each unknown is determined based on the standard curve.

Briefly, the content of one BSA sample was diluted into several vials, preferably using the same diluent solution (usually water) used in the samples, according to the guidelines listed in Table 8.

***Table 8*** *- Dilution scheme for standard test tube protocol and microplate procedure *According to the Pierce™ BCA Protein Assay Kit user guide, Pub. No. MAN0011430 Rev. B.0*

| Vial | Volume of Diluent (µL) | Volume and Source of BSA (µL) | Final BSA Concentration (µL/mL) |
|---|---|---|---|
| A | 0 | 300 µL of stock | 2000 |
| B | 125 | 375 µL of stock | 1500 |
| C | 325 | 325 µL of stock | 1000 |
| D | 175 | 175 µL of vial C dilution | 750 |
| E | 325 | 325 µL of vial D dilution | 500 |
| F | 325 | 325 µL of vial E dilution | 250 |
| G | 325 | 325 µL of vial F dilution | 125 |
| H | 400 | 100 µL of vial G dilution | 25 |
| I | 400 | 0 | 0=blank |

Then, in a 96-well plate, 25 µL of each standard or of each unknown sample was placed into wells. A replicate was done for each one. A working reagent mix was prepared by mixing 50 parts of *BCA Reagent A* with 1 part of *BCA Reagent B* (50:1, A:B), and 200 µL were added into each  each well. After briefly shaking, the plate was covered and left to incubate at 37°C

for 30 minutes. As soon as the incubation time finished, the plate was cooled to room temperature and absorbance measured at 562 nm on the previously referred plate reader. The average 562 nm absorbance measurement of the blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. Then, a standard curve was prepared by plotting the average blank–corrected 562 nm measurement for each BSA standard *vs.* its known concentration in µg/mL. That standard curve was then used to determine the protein concentration of each unknown sample.

### 3.2.2. *Fluorometric Assays to measure the Enzymatic Activity of different Hydrolases*

The rationale underlying all these assays is quite simple: esters of *4-methylumbelliferone* (4-MU) do not fluoresce unless cleaved to release the fluorophore, which emits light at 460 nm when excited by 365 nm light (Figure 9). Thus, by promoting the hydrolysis of 4-MU-containing substrates, and measuring the resultant florescence, it is possible to calculate the activity of the enzyme(s), which promoted the cleavage.

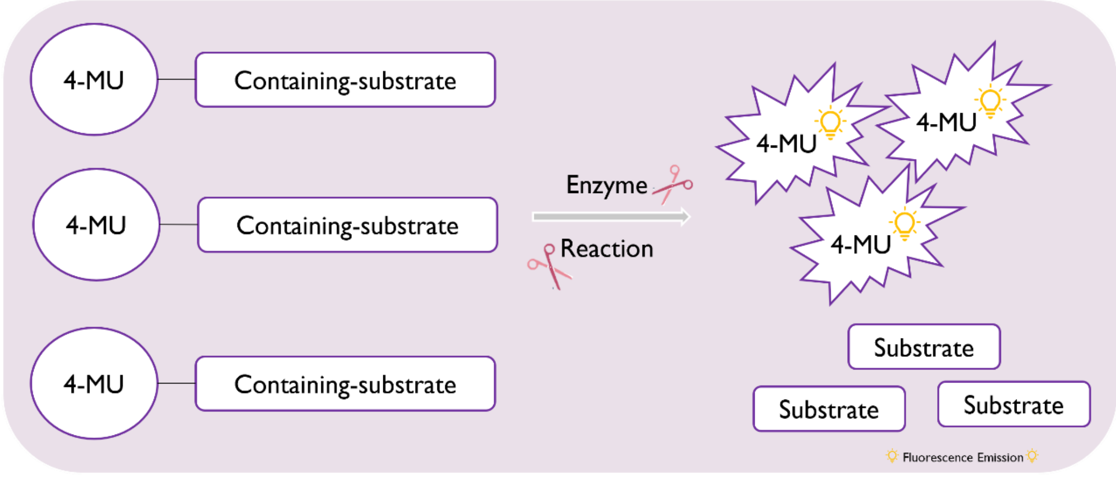



***Figure 9*** *- Principle of fluorimetric assays with 4-methylumbelliferone (4-MU).*

Given their potential to easily and accurately calculate different lysosomal enzyme activities using the exact same quantification protocol in the same instrument, many 4-MU-substrates are currently available in the "*Unidade de Ratreio Neonatal-Departamento de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge*" for diagnostic purposes. So, we took advantage of the availability of those methods to measure several enzyme activities and assess whether: a) the patient-derived SHEDs displayed the enzymatic deficiency, which accounted for their associated pathology and, b) the general values obtained in WT SHED cell lines were comparable to those obtained when measuring the same activities in WT fibroblast homogenates.

The activities measured by this method were the following: iduronate 2-sulfatase (IDS, E.C. 3.1.6.12, the enzyme deficient in MPS II); beta-galactosidase (GLB, E.C. 3.2.1.23, the enzyme

deficient in either GM1-gangliosidosis or MPS IVB); alfa-galactosidase (GLA, E.C. 3.2.1.22, the enzyme deficient in Fabry Disease) beta-glucuronidase (GUSB, E.C. 3.2.1.31, the enzyme deficient in MPS VII); hexosaminidase A (HEXA, E.C. 3.2.1.52, the enzyme deficient in GM2-gangliosidosis) and alpha-N-acetyl-glucosaminidase (NAGLU, E.C. 3.2.1.50, the enzyme deficient in MPS IIIB). Some of them were used as reference.

Bellow, there is a brief overview on all the used 4-MU-based assays and their individual protocols.

Briefly, to prepare the sample replicates, SHED cell homogenates (after sonication) and 4-MU-containing substrate were added simultaneously to 5 mL disposable test tubes or to 96 well-plates (depending of the volumes used). After gentle mixing, the tubes/plates were incubated at 37°C for different times in a slowly oscillating thermomixer or, alternatively, in a pre-warmed water bath. The tubes were then placed in ice, and the reaction stopped with 1000 μL of glycine. Additionally, two blank tubes were assayed for each sample. In general, blanks were prepared by adding 1000 μL of glycine to a mixture of water and of the substrate to be tested, after parallel incubation in the same exact conditions used for the cell samples. The initial volume of SHED cell homogenate, 4-MU-containing substrate, and the incubation times for each enzyme assay were as described on Table 9.

For IDS enzyme activity, though, the standard protocol is not so straightforward: after the previously sonicated cell homogenates were incubated with the respective 4-MU-synthetic substrate (4MU-α-2-sulfate) at 37°C for 4 hours, a second, longer incubation, was also performed, with purified α-iduronidase and only after that second period was the reaction stopped, and fluorescence measured. In the case of IDS, this step is necessary to ensure accurate enzyme analysis. This happens because the enzymatic cleavage of the fluorochrome from 4-MU-α-iduronate 2-sulphate requires the sequential action of IDS and α-iduronidase. However, normal levels of α-iduronidase activity were shown to be_insufficient to complete the hydrolysis of the reaction intermediate 4MU-α-iduronate formed by IDS. A second incubation step in the presence of excess purified α-iduronidase is needed to avoid underestimation of the IDS activity ([229]).

***Table 9*** - *Incubation conditions for the fluorometric lysosomal enzyme assays *According to the methods described by Ciballero et al., 2006* [230]

| Enzyme | Sample Volume (µL) | Substrate | Incubation Time |
|---|---|---|---|
| IDS | 10 | 20 µL of 1.25 mmol/ 4-MU-α-2-sulfate in 0.1 mol/L Sodium acetate / 0.1 mol/L acetic acid buffer + 10 mol/l plumbic acetate, pH 5.0 | Incubation 1: 4 h<br>Incubation 2: 24 h |
| GLA | 10 | 50 µL 5 mmol/l 4-MU-α-D galactoside in 0.15 mol/L citrate-phosphate buffer, pH 4.4 | 1h30m |
| GLB | 10 | 50 µL of 0,8 mmol/L 4-MU- β-D-galactoside in distilled water | 30 min |
| GUSB | 10 | 50 µL of 10 mmol/l 4-MU- β-D-glucuronic acid mmol/l in 0.1 mol/L sodium acetate buffer, pH 4.8 | 45 min |
| HEXT | 10 | 50 µL of 3 mmol/L 4-MU- β-D-glucosaminide in 22 mmol/L citrate-phosphate buffer, pH 4.4 | 15 min |
| HEXA | 10 | 10 µL of 6 mmol/L 4-MU-β-D-N-acetylglucosamine-6-sulfate in distilled water | 1 h |
| NAGLU | 30 | 60 µL of 2 mmol/L 4-MU-2-acetamide-2-deoxy- α-D-glucopyranoside in distilled water | 16 h |

In general, as soon as the reactions were stopped, fluorescence (excitation, 365 nm; emission, 450 nm) was measured in a VICTOR® Nivo™ Plate Reader (PerkinElmer Inc, Waltham, Massachusetts, USA). Readings were corrected for blanks and compared with 4MU calibrators. Enzyme activities were calculated as nanomoles of hydrolysed substrate per hour, per milligram of protein (nmol/h/mg prot).

### *3.2.3. Chromogenic Assay to measure the Enzymatic Activity of Arylsulfatase B*

For ARSB, a different approach was used. In fact, instead of relying on a 4-MU-substrate, this assay used the artificial chromogenic substrate 4-nitrocatecholsulfate to allow for subsequent measurement of ARSB enzyme activity.

This difference may be justified by a simple observation: when the equivalent 4-MU substrate is used to measure enzyme activities in cell homogenates, or even in plasma/leukocytes, there are two different enzymes, which can actually degrade it *in vitro*: ARSB (our target) and arylsulfatase A (ARSA). This means one can easily get an overestimation of the ARSB activity by using that assay.

But, when it comes to the chromogenic substrate 4-nitrocatecholsulfate, it becomes much easier to measure ARSB activity alone. In fact, ARSA hydrolyzes that substrate at 0°C, whereas ARSB is almost inactive at 0°C, hydrolyzing it only at 37°C.

Other than that, the overall protocol for enzyme activity measurement was quite similar to that described for all the other lysosomal hydrolases analyzed (see section 3.2.2), and included a single incubation, at 37°C, for 1 hour.

## 3.3. Assessment of the LSD-associated Subcellular Phenotype(s) in the established MPS patient-derived SHEDs

To assess whether other MPS pathological features apart from the primary enzymatic defect were recapitulated in SHED cell lines, the presence or absence of primary storage products, GAGs, was addressed by LC MS/MS quantification and the existence or not of an abnormal lysosomal pattern by staining the Lysosomal-associated membrane protein 1 (LAMP-1).

### *3.3.1. Glycosaminoglycans (GAGs) quantification by LC MS/MS*

GAGs were quantified by simultaneous analysis of dermatan sulfate (DS) and heparan sulfate (HS) in control (WT) and MPS-derived SHED homogenates, by LC-MS/MS, after butanolysis reaction, according to the method recently described by Forni and co-workers [231,232]. While initially described to perform HS and DS analysis in urine samples, this method was adapted to quantify the same compounds in cell homogenates. Briefly, cell homogenates were prepared by sonication, and their protein concentration determined with the same method described in section 3.2.1. Each individual cell homogenate was divided into two different sample tubes, one for HS and another for DS. Samples were then dried under a stream of nitrogen and 75 µL of 3N *HCl* in *N-butanol* added to each vial. For HS measurements, samples were incubated for 60 min at 90°C. For DS measurements, on the other hand, samples were heated for 25min at 65°C. After those incubations, samples were cooled back to room temperature for 10 minutes and dried under a stream of nitrogen. 100 µL of a 30:70 *water/acetonitrile* (v/v) solution were then added to each HS tube, and 250 µL to each DS tube and briefly vortexed. Finally, the DS samples were combined with their respective HS counterparts and vortexed again. Finally, dimers derived from butanolysis reactions were chromatographed on a HPLC using a gradient of acetonitrile and water (LC column: Gemini® 3µm C6-Phenyl 110 Å,100 x 2 mm, from Phenomenex) and detected on a triple quadrupole mass spectrometerAPI4000 QTRAP from Sciex. Samples were quantified by interpolation from the calibration curve (prepared to cover a concentration range from 0.39 to 50 µg/mL

for HS and from 1.56 to 100 µg/mL for DS using seven different dilutions) and reported in mg/mL. Then, HS and DS were normalized to protein concentration.

### 3.3.2. *LAMP-1 Immunocytochemistry*

Lysosomal accumulation, which is a common feature to many LSDs, is frequently associated with an increase in LAMP-1 expression. To evaluate if this feature was noticeable in MPS-derived SHED cell lines, a LAMP-1 immunocytochemistry assay was performed, always comparing with control SHEDs.

LAMP-1 staining is an immunocytochemistry assay, and therefore similar to the above described for neuronal markers, with a few adaptations. Before the fixative solution was applied, 3 washes were performed with *PBS 1X*. In fact, every step of this procedure was followed by 3 rounds of washes with *PBS 1X*. Furthermore, the composition of the fixative solution was *Paraformaldehyde (PFA)* 4% in *PBS 1X*, with an incubation time of 30 minutes. Outside laminar flow, an incubation with $NH_4Cl$ was performed for 10 minutes at room temperature. The permeabilization reagent used was *Methanol Ice-cold* with an incubation of 10 minutes at room temperature. Then, as a blocking solution, *5%BSA/PBS 1X* was used and incubated for 60 minutes at room temperature.

The primary antibody, *Anti-LAMP-1-H4A3* (raised in mouse, monoclonal; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), was applied over 90 minutes in a 1:200 dilution in *BSA/PBS 1X*. The second one, Alexa Fluor 488 (goat anti-mouse; Thermo Fisher Scientific, Waltham, Massachusetts, USA) was applied after 3 washes with PBS 1X, for approximately 45 minutes in a 1:1000 dilution.

Again, a wash step was performed with PBS 1X and then with water, always protected from light. For the assembly step, the Mount-Mowiol with DAPI mounting medium was applied with the lamellae. Before the acquisition, the sample was left to dry protected from light for at least for 90 minutes.

# Results

# *Results*

## 1. Establishment of a protocol for primary cell culture of Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) *in house*

After a successful call for healthy volunteers, numerous families requested for a 'tooth collection kit' for normally shed deciduous teeth, or SHED. Thus, over 50 kits were carefully prepared and sent by regular mail, as described in the *Materials and Methods*, section 1.1.2.

Over the following period, around 40 of those kits were returned to the laboratory, carrying, each one, its own deciduous tooth and only those that showed no signs of medium contamination were kept for primary SHED cell culture. Overall, the teeth included in the study were non-carious, had no previous restorations, and had no reports of prior trauma, even though two were surgically extracted (both donated from the same child). All other samples were spontaneously exfoliated teeth. Successful primary cultures were established for more than 30 controls.

In general, cell adhesion took around 1-2 weeks, but as soon as the first cells adhered, the proliferation rate started to rise exponentially, and usually at two and a half weeks the cells were already confluent. As depicted in Figure 10, SHEDs grow in an adherent way and display a normal morphology characterized by a spindle shape, similar to fibroblasts (Figure 10A), which initially formed small colonies (Figure 10B-C) that were left to grow up until they reached sub-confluency (Figure 10D).

Throughout the whole process, which involved the establishment of the primary cultures, their passage, freezing and thawing, cells viability and morphology were checked on a daily basis and every relevant alteration noted. In general, all established cell lines shared the same fibroblast-like morphology, which remained unaltered for several passages. For a few control cell lines, primary cultures were kept for over 10 passages, without any significant morphological change. Also noteworthy, their proliferation rates remained significantly higher than those observed for fibroblast cell lines (namely, for HDFa, a commercial adult human dermal fibroblast cell line, which is routinely used *in house*). Altogether, the teeth received under the so-called "*2020's Tooth Fairy Project*", allowed for a careful optimization of all the reagents, conditions and methods, which contributed for the successful establishment and maintenance of this sort of cultures *in house*.

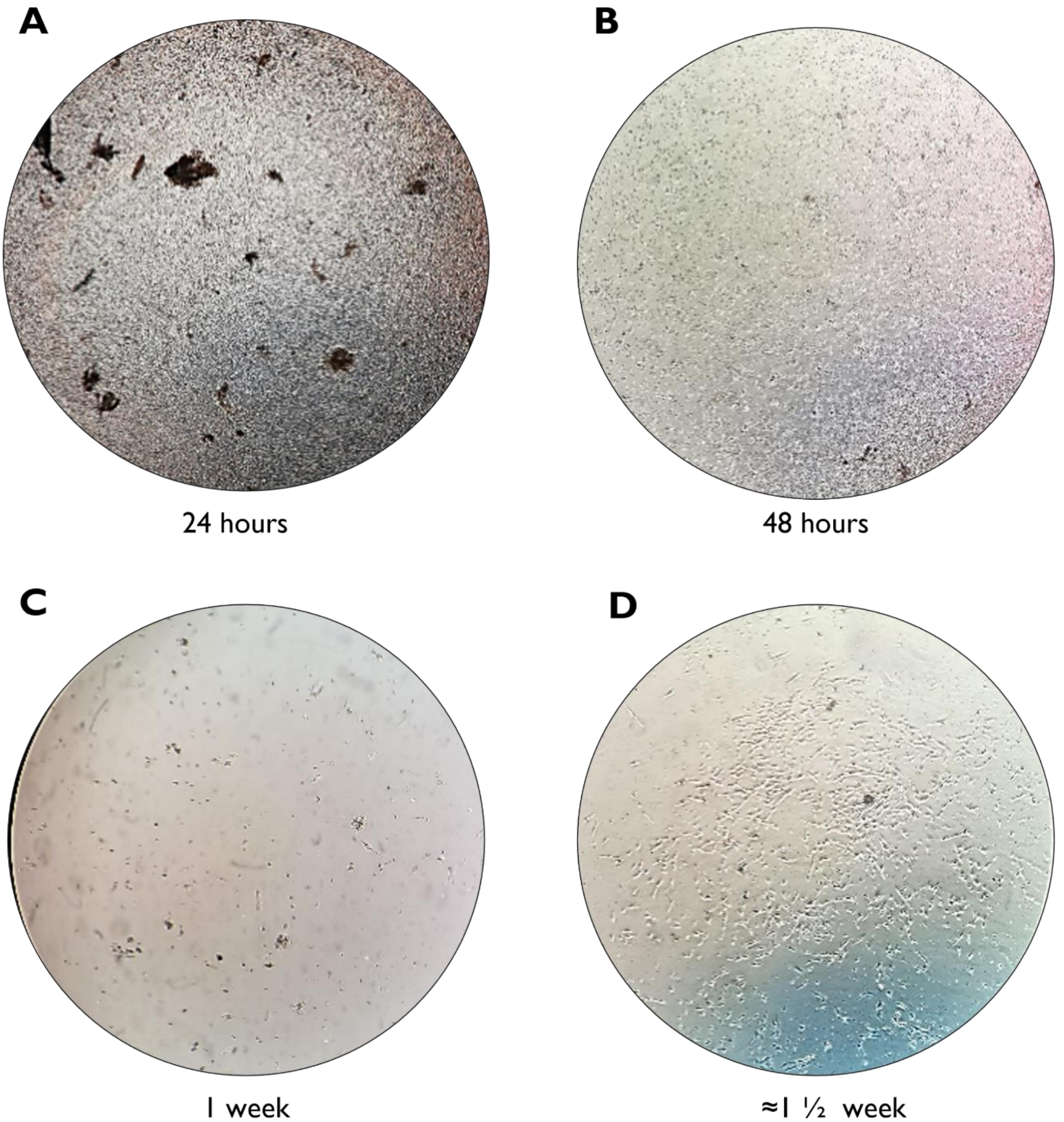



***Figure 10*** *- Isolation of SHEDs from dental pulp and establishment of the primary culture. (A) Typical pulp culture on day one, with a lot of visible debris (in black); (B-D) Culture expansion in a selective medium (DPSC Culture Medium), which promotes the selective adhesion of cells of interest. Bellow every scheme, there is a reference to the time (hours/days/weeks) post dental extraction protocol.*

## 1.1. Confirmation of the stemness potential of control SHED cell lines

While all observations regarding cells' adhesion, morphology and proliferation rate reported so far were consistent with the assumption that those were indeed stem cells from human exfoliated deciduous teeth, i.e., SHEDs, before MPS patients started to be recruited, it was mandatory to actually confirm the stemness potential of those cells.

Therefore, a pilot qRT-PCR analysis was performed in two randomly picked controls, to quantify the expression of three major pluripotency markers.

#### *1.1.1. Quantitative analysis of the expression of three pluripotency markers*

To confirm the pluripotency of the established cell lines over several passages (px2; px5; and px7) the quantitative expression of three known pluripotency markers was evaluated: *Nanog*, *OCT 3-4*, and *Sox-2*. As seen in graphics from Figure 11, the results were positive for all markers, in all analyzed passages. Furthermore, the results show that, at least for the passages assessed, the higher the passage, the higher the expression of the evaluated pluripotency markers.

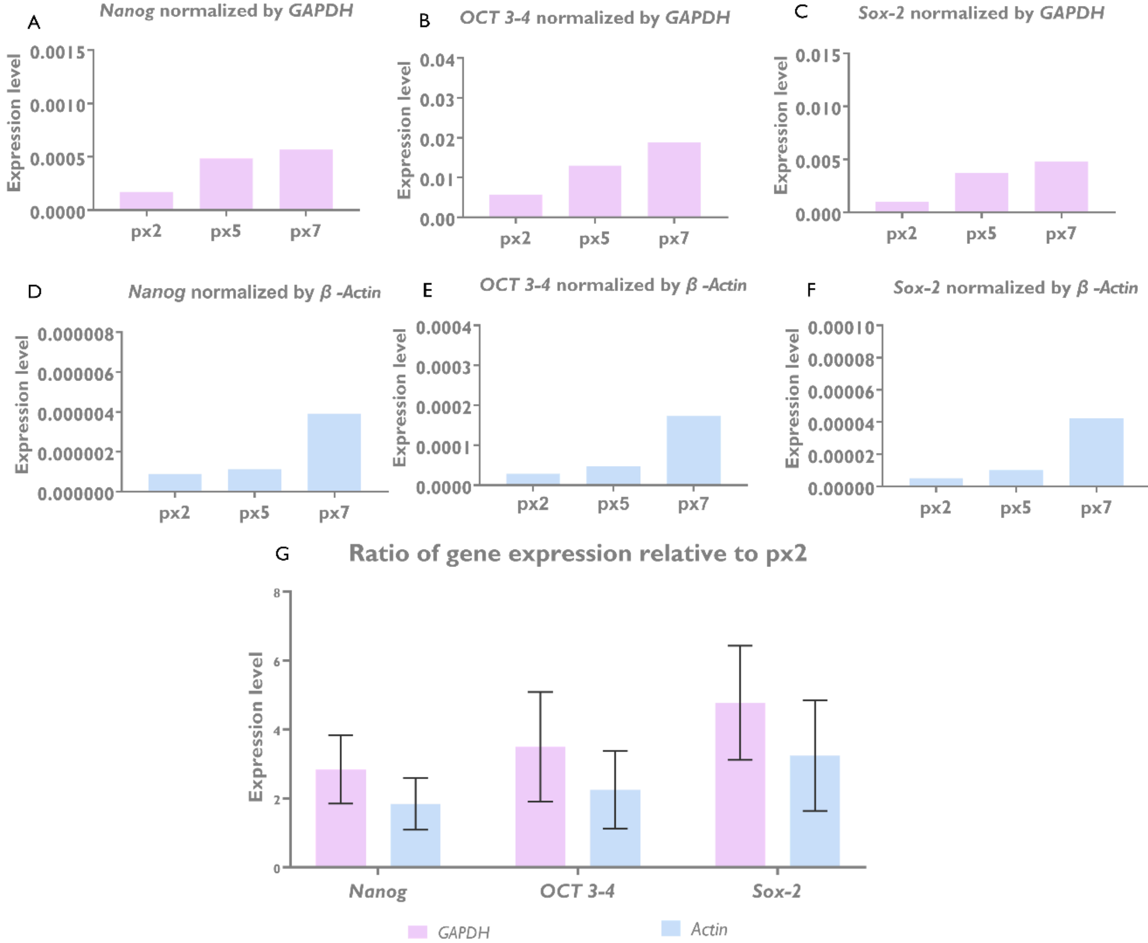



***Figure 11*** *- Real-Time PCR analysis of pluripotency markers. (A), (B), and (C)* Nanog, OCT 3-4, *and* SOX2 *(respectively) expression levels, normalized to* GAPDH. *(D), (E), and (F) Nanog, OCT 3-4, and SOX-2 (respectively) expression levels, normalized to* β-actin. *Results are shown for passages px2, 5, and 7, respectively. (G) Ratio of expression of px7 relatively to px2, with* β-actin *and* GAPDH *normalization.*

As soon as the presence of pluripotency markers was confirmed in these cells, the protocol was considered validated, and the call for volunteers extended to MPS patients and their families, under the terms and conditions described in the *Materials and Methods* chapter, section 1.1.1. (*Call for volunteers*).

## 2. Collection and isolation of both MPS patient-derived SHEDs for primary cell culture establishment

In order to facilitate the access of the higher possible number of patients to this project, a few dozen 'tooth collection kits' were prepared and distributed among the pediatricians who are involved in this project. From those, only three have returned to the laboratory, allowing for the successful establishment of an equal number of unrelated MPS-derived SHED cell lines.

Briefly, three different disease cell lines were established: two from MPS II patients and the third from an MPS VI patient. According with the age of onset of the first symptoms and the clinical data sent by the responsible clinicians, all three patients had severe forms of the disorders Table 10.

***Table 10*** *- Clinical data from MPS II and MPS VI patients sent by the responsible clinicians, including, age of diagnosis, symptoms and age of starting treatment.*

| Disease | Case | Age of diagnosis (years) | Symptoms | Age of starting treatment (years) |
|---|---|---|---|---|
| MPS II | 2.01 | 3 | Coarse facies,, Stiff Joints, etc.<br>Post-natal macroglossia<br>Mild psychomotor development retardation;<br>Interventricular communication (IVC) and patent ductus arteriosus (PDA), but solved by now;<br>Moderate aortic unsufficiency and left ventricular hypertrophy;<br>Hydrocele;<br>Chronic nasal obstruction without recurrent otitis or hearing deficit. | |
| MPS II | 2.02 | 2 | Inguinal Hernias;<br>Claw Hands;<br>Low stature;<br>Hypertrichosis;<br>Hepatomegaly;<br>Cardiac involvement. | 4 |
| MPS VI | 6.01 | 8 | Hepatomegaly;<br>Voluminous umbilical Hernia;<br>Short Stature;<br>Dysostosis;<br>Cardiac involvement;<br>Respiratory Failure. | 8 |

Naturally, the teeth from which those cell lines were established were received over time during the course of this thesis, whenever an eligible patient lost a deciduous tooth. This means that some of the results described henceforward were not obtained in parallel or simultaneous assays but performed individually, instead.

## 3. Confirmation of the stemness potential of the established MPS SHED cell lines and validation of their Mesenchymal Stem Cell phenotype

### 3.1. Quantitative analysis of the expression of standard pluripotency markers and other specific surface antigens

As soon as the first MPS cell lines were established in the laboratory, it became mandatory to confirm their stemness potential by assessing whether it was possible to amplify, by qRT-PCR, the pluripotency markers previously assessed in control cell lines (*Nanog*, *OCT 3-4*, and *Sox-2*). However, instead of assessing their expression levels in different passages, investing in understanding how the culture behaved over time, it was considered far more relevant to further expand the catalogue of quantified markers to check the expression levels of additional cell surface antigens. In fact, as reported in the *Introduction* section, there are certain specific surface antigens whose presence (or absence, in a few cases) strongly correlates with MSCs identification, and so, their quantitative analysis becomes relevant for a proper cell line characterization. Therefore, those markers were also included in the qRT-PCR assay, whose results will now be described.

In the qRT-PCR assay, in addition to the MPS samples, 4 distinct controls, all derived from the received baby teeth, were also included. Additionally, also an iPSC sample derived from Fabry disease fibroblasts[2], got included, as it would allow for comparisons with a previously reported and well-characterized stem cell line [233].

The results, which are summarized in

Table 11 as mean ΔCts, clearly demonstrate similarity among all the different cell lines analyzed, when it comes to specific MSC cell surface antigens. In fact, the specific MSCs markers (*CD105*, *CD73*, and *CD90*) are the ones that present the lower ΔCt values, which

[2] Induced pluripotent stem cell line from a Fabry Disease patient hemizygote for the rare p.W287X mutation (INSAi002-A); this is a stem cell resource, kindly provided by AJ Duarte et al. [233].

correlate to higher expression levels of those genes/proteins. When the results for those three markers were compared to the ΔCt values obtained for two other cell surface markers whose expression is not typical of MSCs: *CD34* (usually associated with lymphohematopoietic stem cells, and endothelial cells) and *MHCII* (normally found only on professional antigen-presenting cells), the MSC phenotype becomes even more evident, with these last two markers presenting much higher ΔCts (therefore less expression; Figure 12A). While no previously characterized MSC line was available in the lab, which could be enrolled as a positive control for characteristic phenotype, the included iPSC sample further validated the obtained results, as it presented almost the same expression pattern for these MSC-related cell surface antigens as the SHED cell lines established under the scope of this thesis.

Looking in particular the expression levels of the pluripotency markers (*Sox-2*, *Oct3-4*, and *Nanog*), all SHED cell lines presented with positive, yet weak expression levels, as reported for DMSCs in general. On the other hand, in the iPSC sample, the detected ΔCts for *Sox-2*, *Oct3-4*, and *Nanog* markers were much lower, further confirming the prominent pluripotency character of those cells (Figure 12B). In fact, this is an expected result, once iPSCs are reprogrammed to overexpress those markers. Contrarily, MSCs despite presenting the possible expression of pluripotency markers, are "one step forward" when it comes to the potential stage. Altogether, these data provide strong evidence on the MSC nature of the established cell cultures, and it is accordance to which is described in literature for various types of Dental MSCs, including SHEDs.

***Table 11*** *- qRT-PCR results of several markers, including* CD105, CD73, *and* CD90 *(MSCs markers),* Sox-2, OCT 3-4, *and* Nanog *(Pluripotency markers),* CD34, *and* MHCII, *in SHEDs from patients and controls, and also iPSCs derived from Fabry fibroblasts. Differences were calculated using the standard ΔCt methods, with* GAPDH *and* β-actin *as housekeeping genes.*

| Target Gene | | Cell lines | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MPSII (2.01) | MPSII (2.02) | MPSVI | Ct1 | Ct2 | Ct3 | Ct4 | iPSCs |
| *CD105* | ΔCt | 5.42 | 4.95 | 4.97 | 5.76 | 5.21 | 5.29 | 4.78 | 7.26 |
| *CD73* | ΔCt | 5.01 | 6.45 | 3.99 | 4.36 | 4.85 | 4.06 | 5.95 | 5.87 |
| *CD90* | ΔCt | 3.96 | 3.69 | 2.18 | 4.42 | 3.37 | 3.56 | 3.32 | 1.73 |
| *Sox-2* | ΔCt | 19.71 | 20.56 | 19.94 | 20.84 | 19.29 | 20.17 | 19.82 | 11.10 |
| *OCT3-4* | ΔCt | 12.92 | 12.60 | 13.35 | 13.28 | 12.98 | 12.83 | 13.09 | 8.02 |

| *Nanog* | ΔCt | 15.84 | 16.07 | 15.99 | 20.10 | 14.74 | 15.19 | 14.44 | 8.88 |
|---|---|---|---|---|---|---|---|---|---|
| *CD34* | ΔCt | 15.65 | 18.49 | 16.72 | 20.86 | 16.96 | 18.86 | 17.12 | 12.93 |
| *MHCII* | ΔCt | 13.09 | 9.63 | 11.26 | 17.34 | 16.72 | 16.22 | 12.47 | 10.32 |

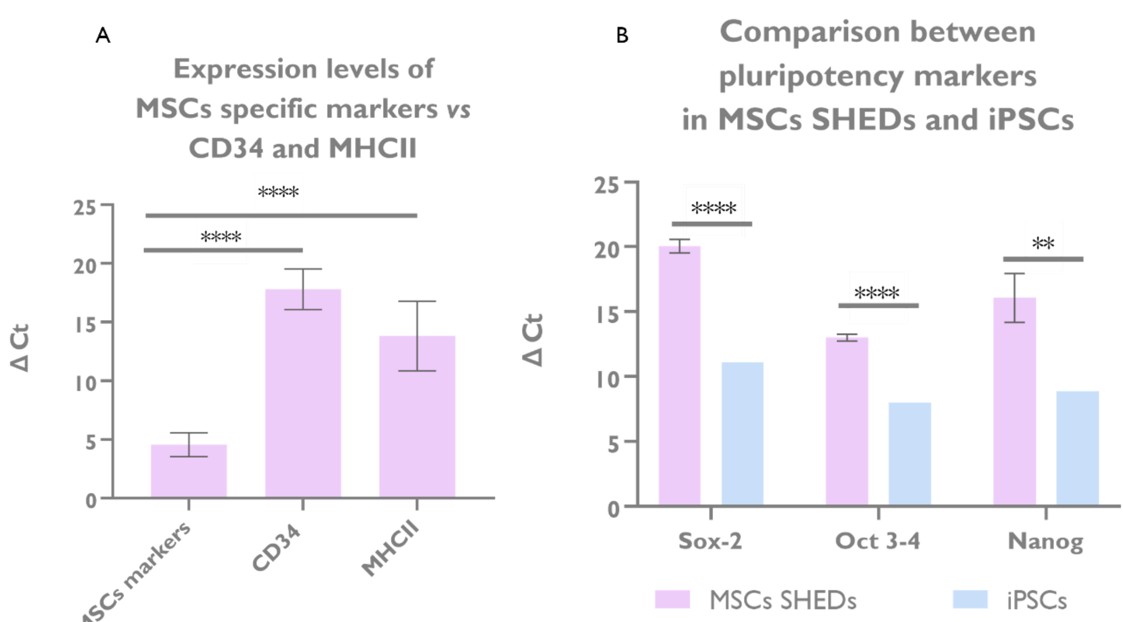



***Figure 12*** *- qRT-PCR results statistically treated. (A) Comparison between MSCs specific markers (CD105, CD73, and CD90) and CD34, and between MSCs specific markers (CD105, CD73 and CD90) and MHCII, in SHEDs. A mean of all ΔCt results obtained for the three different MSCs markers measured in SHEDs cell lines was compared to the mean of ΔCt results for CD34 and MHCII markers, in the same cells. (B) Pluripotency markers expression in MSCs SHEDs vs iPSCs in both cases, significant differences were found: **** $p<0.0001$; ** $p<0.05$.*

### 3.2. *In vitro* multilineage differentiation into different cell types

Traditionally, one of the listed requirements to identify MSCs is their ability to differentiate into three different cell types: adipocytes, osteocytes and chondrocytes. More recently, though, many authors have argued those requirements should be updated to include cells from the 3 germ layers: ectoderm, mesoderm, and endoderm.

Therefore, in this work, apart from the classical differentiation protocols (into adipocytes, osteocytes and chondrocytes, which derive from the mesoderm), a fourth protocol was also included: neurogenesis, to validate the overall capacity of these cells to differentiate from the ectodermal germ layer.

There are some fundamental technical differences between the protocols for adipogenesis, osteogenesis and chondrogenesis, and that of neurogenesis, as described in the *Materials and Methods* chapter, section 2.2.. Therefore, the results from the first three differentiation methods were grouped together and will be discussed as a whole, while those from the neurogenesis protocol, will be addressed on their own. Regarding adipogenesis, osteogenesis and chondrogenesis, after the recommended 21 days of differentiation, the morphological changes in cells subjected to chondrogenic and osteogenic procedures were evident (Figure 13B and C), while no significant morphological differences could be observed on the cells subjected to adipogenic differentiation (Figure 13D). It should be stressed, however, that the referred morphological changes were observed under light microscope. This means that, while drastic morphological changes such as those observed in the cells subjected to osteogenesis and chondrogenesis, may be easily documented, the same may not apply to those under an adipogenesis protocol because the morphological differences between SHEDs and adipocytes are much less obvious. It can not be ruled out the possibility that, those same cells, observed by electron microscopy, for example, would appear significantly different.

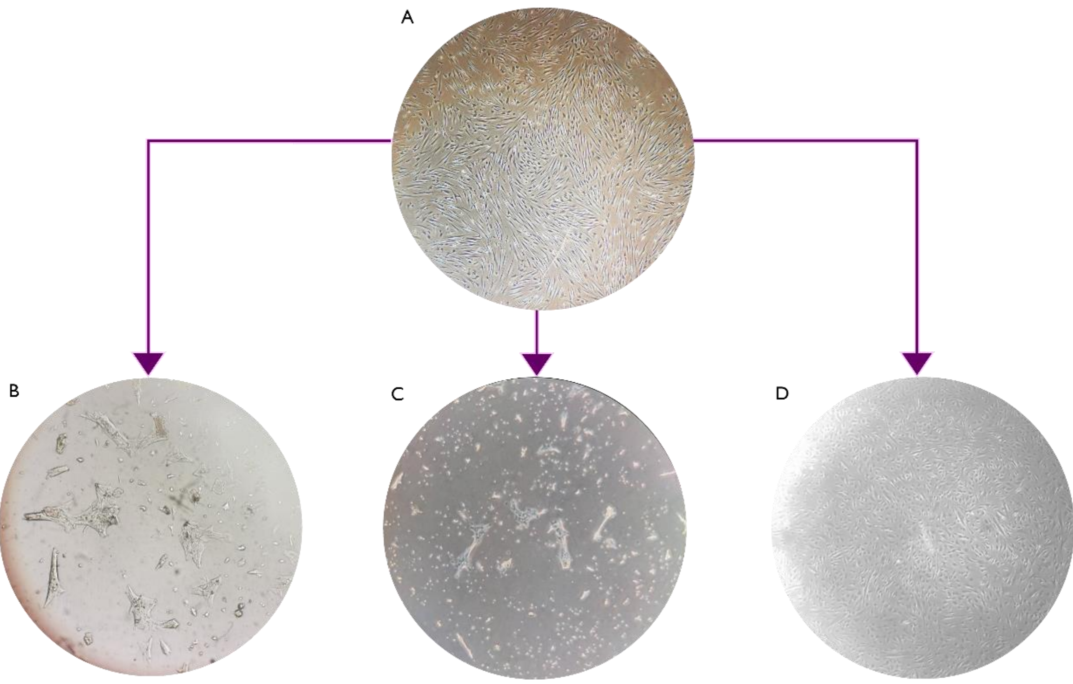



***Figure 13*** *- Differentiation potential of SHEDs. The primary cultures of patient-derived SHEDs (A) were treated for 21 days under (B) chondrogenic, (C) osteogenic and (D) adipogenic conditions.(A) Low magnification image of seeded SHEDs prior to the experiment (B) Chondrogenic differentiation, with significant morphology changes; (C) Osteogenic differentiation with significant morphology changes; (D) Adipogenic differentiation with no perceptible morphological differences.*

As described, the protocol includes three stages: Neural Induction, Neural Expansion, and Neuronal Differentiation.

During the Neural Induction stage, the cells behaved normally, without significant morphological changes observed (Figure 14B). However, in stage two, which was supposed to correspond to an expansion stage, the cells seemed to reach a "stationary phase" instead (Figure 14C). In fact, virtually no cell proliferation was observed for several days, and even after one subsequent passage. No extra passages were performed and then, the third and final medium, that induces neuronal maturation, was applied. By then, it was possible to observe a significant cell mortality rate. Still, a few days later, the remaining cells started to present significant morphological changes, reaching a final morphology consistent with that of typical neurons: a pyramidal-like soma with shorter projections similar to dendrites and a longer axonal projection on the opposing side (Figure 14D). Also noteworthy, none of those neuron-like cells showed any proliferation capacity. In fact, even though maintained for over three weeks with regular medium changes, none of those cells was able to divide, an observation that is fully compatible with the assumption that the SHEDs under analysis have actually differentiated into neurons.

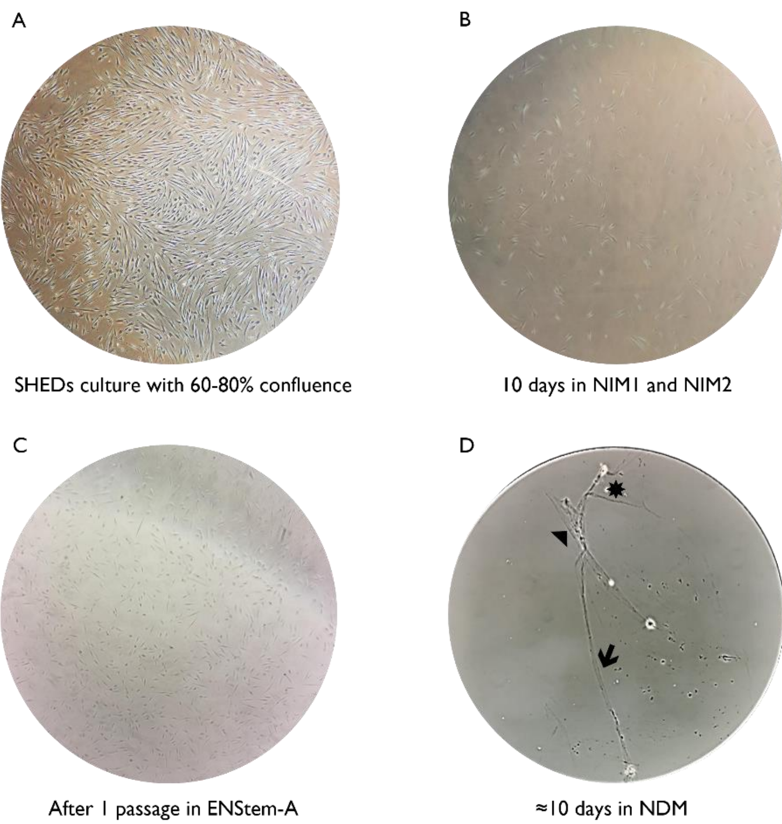



***Figure 14*** *- First attempt of SHEDs' Neurogenic Differentiation. (A) SHED cell culture with 60-80% confluence, immediately before the neurodifferentiation protocol was initiated; (B) Neuronal Induction stage; (C) Neural Expansion stage; (D) Neuronal Cell with a pyramidal-like soma (▲) with shorter projections similar to dendrites (✱) and a longer axonal projection (🡇).*

## 4. Assessment of the presence of neuronal markers in the established SHED cell lines

As already referred in *Materials and Methods* section 2.3., while not initially envisaged, an additional characterization protocol was also performed: an immunocytochemistry assay to confirm the DMSC early commitment to their so-called "neuronal fate". And in fact, staining of neuronal markers in SHEDs not subjected to any type of neurodifferentiation protocol, revealed a positive fluorescence pattern for all four markers evaluated: Nestin; Sox-1; Pax-6 and Sox-2 (Figure 15), further validating the assumption that SHEDs may actually be classified as Neural Progenitor Cells (NPCs), as stated by several different authors.

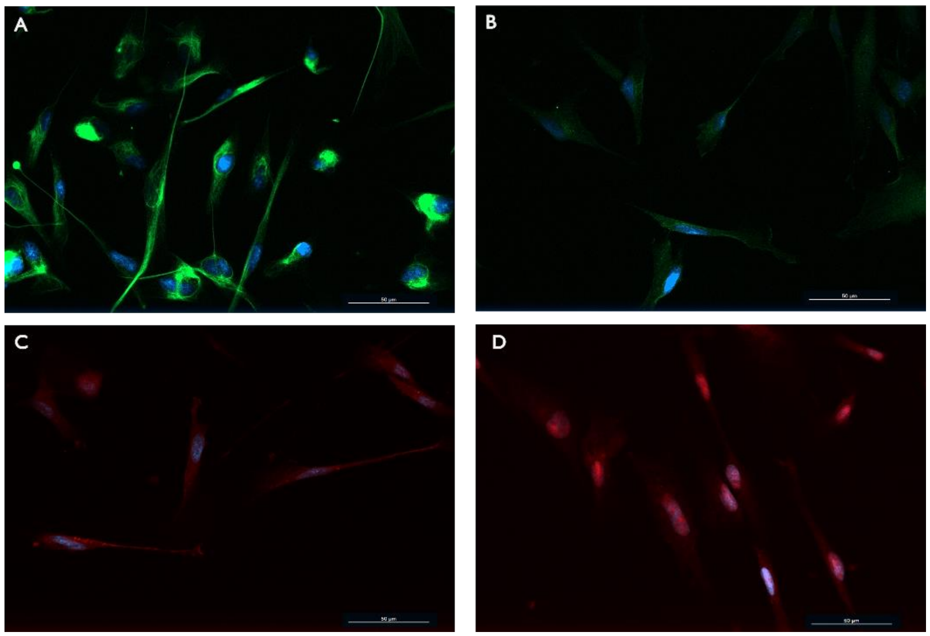



***Figure 15*** *- Neuronal markers immunostaining in SHEDs. All of the markers analysed (A:Nestin; B: Sox-1; C: Pax-6; D: Sox-2) show a well-marked fluorescence in SHEDs, confirming the expression of those protein markers.*

## 5. Molecular and biochemical characterization of the established MPS patient-derived SHEDs

### 5.1. Molecular and biochemical confirmation of the disease-causing enzymatic defect(s) in each established cell line

Whenever an MPS tooth was received in the laboratory, the only information it brought along was the type of MPS from which the patient it belonged suffers. Therefore, as soon as its derived SHED cell line was established and the first vials stored, cell pellets were collected and used for mutational analysis, as described in the *Materials and Methods* chapter, section 3.1. For each patient, only the gene, which underlies his/hers associated disorder was

sequenced. The results from all the performed mutational studies are summarized in the next paragraphs.

### *5.1.1. Mucopolysaccharidoses type II*

For the two MPS II cases, the underlying mutations were found on the *IDS* gene, which encodes Iduronate 2-Sulfatase (IDS).

#### *5.1.1.1. Case 2.01*

For the first MPS II case, the molecular characterization was a bit challenging. In fact, after amplifying all *IDS* exons and their surrounding intronic regions and analyzing the resulting electropherograms, an apparent unchanged sequence was observed.

Therefore, cDNA was amplified to check for the possible presence of splicing pattern alterations, as a result of deep intronic or other previously unanticipated pathogenic variants, since it is well-documented that alternative splicing at the *IDS* gene is very common due to the presence of numerous cryptic splice sites within the gene [227]. Interestingly, it was not possible to amplify any of the cDNA fragments.

It became, then mandatory to revisit the literature on molecular characterizations of MPS II patients. And, by reading the results of the molecular characterization of Portuguese patients suffering from this disorder, a paper which was originally published by the R&D group where this thesis was carried out [227], one particular mutation seemed to be consistent with our observations so far: a rearrangement involving recombination between intron 7 of the *IDS* gene and sequences located distal of exon 3 in the *IDS* pseudogene (*IDS-2*). This recombination event had already been reported, and is known to cause a partial inversion of the *IDS* gene [234]. So, a previously described restriction fragment length polymorphism (RFLP) assay was conducted to specifically look for the presence of that mutation. Briefly, amplicons generated with the primers previously reported by [235] were incubated with the *Hinf* I restriction enzyme, as reported by Lagerstedt et al. Through that simple analysis, it would be easy to distinguish between cases, which harbor the rearrangement, and others that do not. Following RFLP analysis, the digested amplicons were observed by electrophoresis in 2% agarose gel and the results confirmed the presence of the suspected rearrangement: GAATC>AGAGG (IDSP1> IDS) (Figure 16).

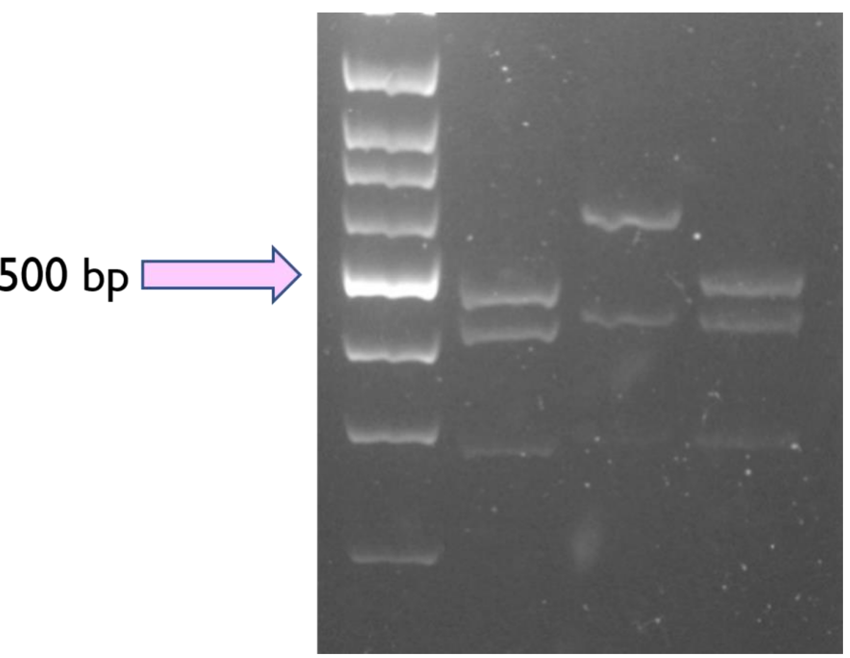



***Figure 16*** *- Identification of recombinants by RFLP analysis with HinfI on products of the PCR amplification with primers gIDS PJ 1F and gIDS PJ 1R, according to the method described by Lualdi et al* [235]*.. As a consequence of GAATC>AGAGG (IDSP1> IDS) mismatches occur, causing the loss of one HinfI site. Therefore, enzyme digestion of the PCR product obtained for Case 2.01 produces a different pattern from that of digested amplicons from a control (Ct) and from the other MPS II patient enrolled in this study: Case 2.02.*

#### *5.1.1.2. Case 2.02*

On case 2.02, on the other hand, the causing mutation was much easier to detect. In fact, when the electropherograms were first analyzed, a single variant was detected in hemizygosity (Figure 17): the previously reported c.22C>T (p.R8*) nonsense mutation [236]. Nonsense mutations are single nucleotide variations within the coding sequence of a gene that result in a premature termination codon (PTC). The occurrence of such PTCs most often leads to a complete loss of protein function and a reduction in mRNA levels due to the nonsense-mediated mRNA decay (NMD), a cellular surveillance mechanism that triggers selective degradation of mutant transcripts [237]. In general, nonsense mutations tend to be associated with severe phenotypes, as the one presented by this patient, who was diagnosed at 2 years old. This mutation had already been reported in different populations, correlating either with severe or intermediate forms of the disease [236,238,239].

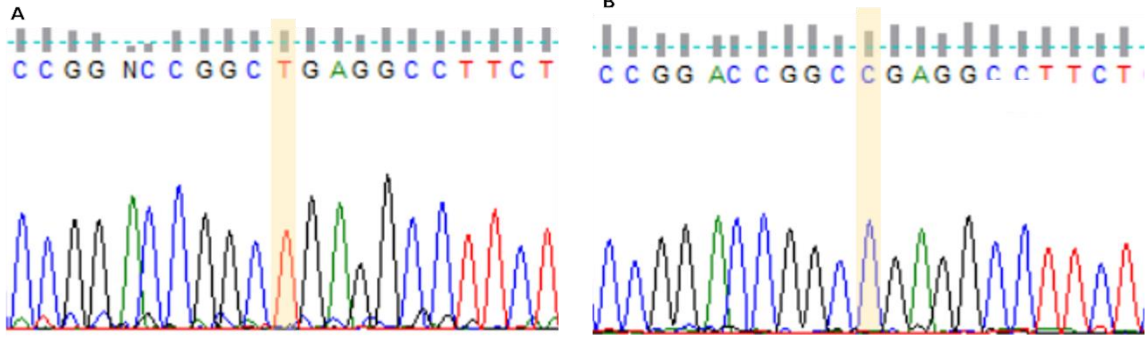



***Figure 17*** *- Molecular analysis of the IDS gDNA by Sanger sequencing. Electropherogram showing part of the exon 1 highlighting the affected base (A) in the patient carrying the c.22C>T variant and (B) in a control.*

### *5.1.2. Mucopolysaccharidoses type VI*

For the MPS VI sample, the sequenced gene was *ARSB*, the one responsible for encoding the lysosomal enzyme arylsulfatase B (ARSB), which is either missing or dysfunctional in that disorder, as already described in the *Introduction* section. The initial molecular study was performed in genomic DNA and only after all exons and their respective intronic boundaries were sequenced and the variants identified, the cDNA analysis was carried out.

For the MPS VI case, the molecular characterization was quite straightforward. In fact, when the electropherograms were first analyzed, two variants were detected in homozygosity: c.971G>T and c.1362G>A, located on exon 3 and 4, respectively. However, when translated to protein, the c.1362G>A modification was shown to be silent (p.P454P). The c.971G>T variant (Figure 18), on the other hand was predicted to give rise to an amino acid exchange, a glycine by a valine (p. G324V). It would probably be the disease-causing allele in this patient. A brief search on the Human Genome Mutation Database (https://www.hgmd.cf.ac.uk/ac/index.php) and in the literature further supported this assumption, as this variant had already been reported in two independent patients. In fact, it was originally reported in a compound heterozygous MPS VI patient, and is known to cause the disease ever since, even though its effect was not totally understood then [240]. Later, it was also reported in homozygosity and classified severely pathogenic, as suggested by the rapidly progressive phenotype of the patient in which it was found. In fact, this pathogenic variant was present in an individual who had macrocephalia, hepatosplenomegaly, severe joint

deformities and eye problems at a very young age [241]. The presence of this mutation was confirmed at cDNA level.

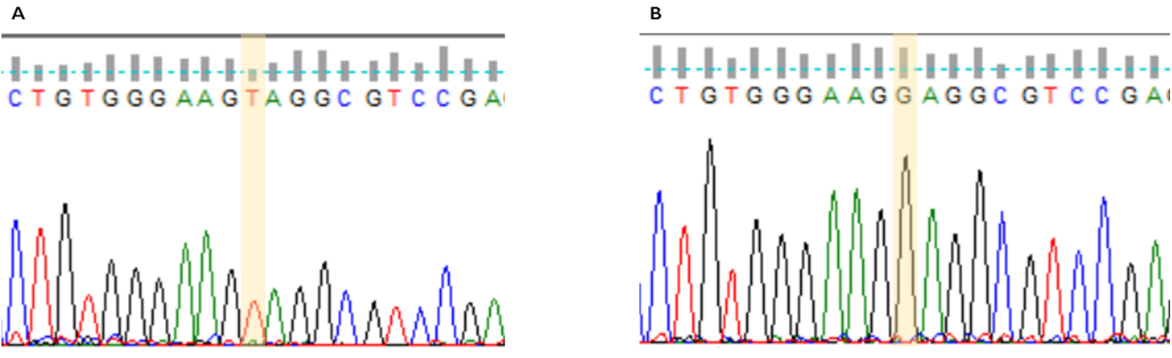



***Figure 18*** *- Molecular analysis of the ARSB gDNA by Sanger sequencing. Electropherogram showing part of the exon 3 highlighting the affected base (A) in the patient carrying the c.971G>T variant and (B) in a control.*

### 5.2. Enzymatic Activities

As soon as the molecular defects underlying the different MPSs enrolled in this study were unveiled, their associated biochemical defects were also addressed. Therefore, the enzymatic activity levels of ARSB were measured in the MPS VI patient, while those of IDS were measured for both MPS II cases, again in SHED cell pellets.

As expected, all enzymatic defects were confirmed. In fact, ARSB activity was significantly decreased in the MPS VI cells (Figure 19A) and IDS was null in both MPS II patients (Figure 19B). All the values obtained are consistent with severe forms of both diseases, with early onset phenotypes, as those seen in the three patients.

Altogether, these results further validate the SHED cell model also presents the enzymatic defects seen in other cell lines more frequently used for the characterization or even diagnosis of different LSDs, such as fibroblasts.

***Figure 19*** *-Enzyme Activities differences between controls (n=20) and MPS-derived SHEDs confirming the enzymatic defects of (A) Iduronate 2-Sulfatase in the two MPS II patient cell lines and (B) Arylsulfatase B in the MPS VI-derived cell line. **** p>0.0001.*

Apart from the logical assessment of disease-associated enzymatic defect in its respective SHED cell line, during the course of this work, it was also considered relevant not only to understand whether the values obtained in WT and MPS SHED cell lines were comparable for other lysosomal enzymes but also whether the values obtained for SHEDs in general, were comparable to those usually obtained for fibroblasts. Therefore, other lysosomal enzyme activities namely alfa-galactosidase (GLA, E.C. 3.1.2.22, the enzyme deficient in Fabry Disease); beta-galactosidase (GLB, E.C. 3.2.1.23, the enzyme deficient in either GM1-gangliosidosis or MPS IVB); beta-glucuronidase (GUSB, E.C. 3.2.1.31, the enzyme deficient in MPS VII); total hexosaminidase (HEXT); hexosaminidase A (HEXA, E.C. 3.2.1.52, the enzyme deficient in GM2-gangliosidosis), and; alpha-N-acetyl-glucosaminidase (NAGLU, E.C. 3.2.1.50, the enzyme deficient in MPS IIIB) were also measured in control (WT) and/or MPS-derived SHEDs. For these particular studies, a higher number of controls was used (n=20), to account for the individual differences, which were already known, from the measurements in fibroblasts and leukocytes, to be significantly high. As seen in Figure 20, lysosomal enzymes not directly involved in respective pathologies show a similar level between WT and MPS SHEDs. Additionally, in general, all measured enzymes were readily detected with the standard methods in use for leukocytes and/or fibroblast homogenates, and their levels were comparable to those obtained for fibroblasts in routine diagnosis (*data not shown*).

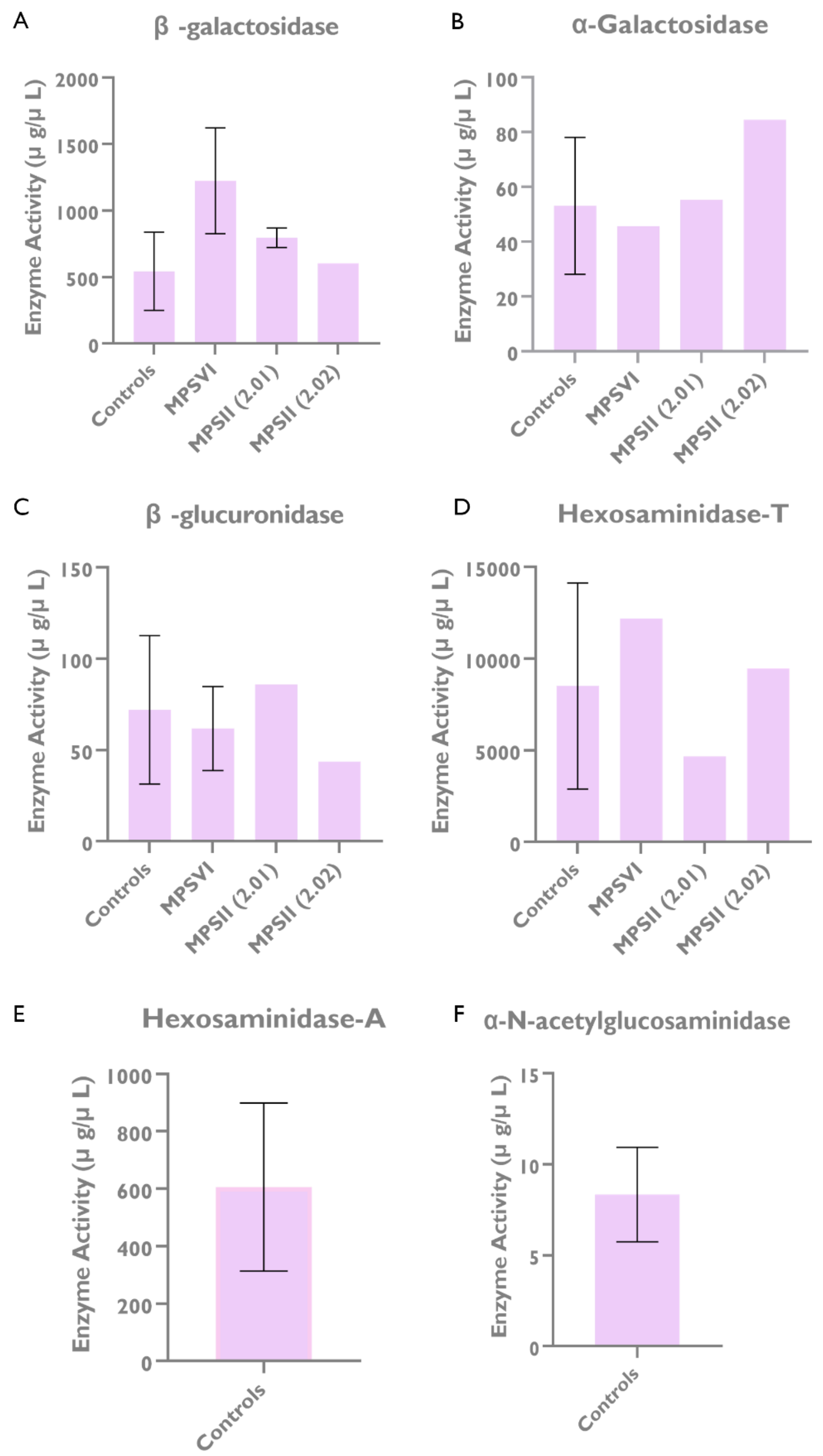



***Figure 20*** *- Enzymatic activities in and/or MPS-derived SHEDs for several lysosomal enzymes.*

## 5.3. GAGs accumulation

The enzyme deficiencies, which underlie MPSs promotes an accumulation of undegraded or partially degraded GAGs. Overall, that accumulation phenomenon is another hallmark of these disorders.

In the case of MPS II and MPS VI the GAGs, which accumulate are dermatan sulfate (DS) and heparan sulfate (HS) (as referred in *Introduction* section). Therefore, by measuring the cellular content of those two substrates in WT and MPSs SHED, it was possible to confirm the GAG storage phenotype in all established MPS-derived SHED cell lines (Figure 21).

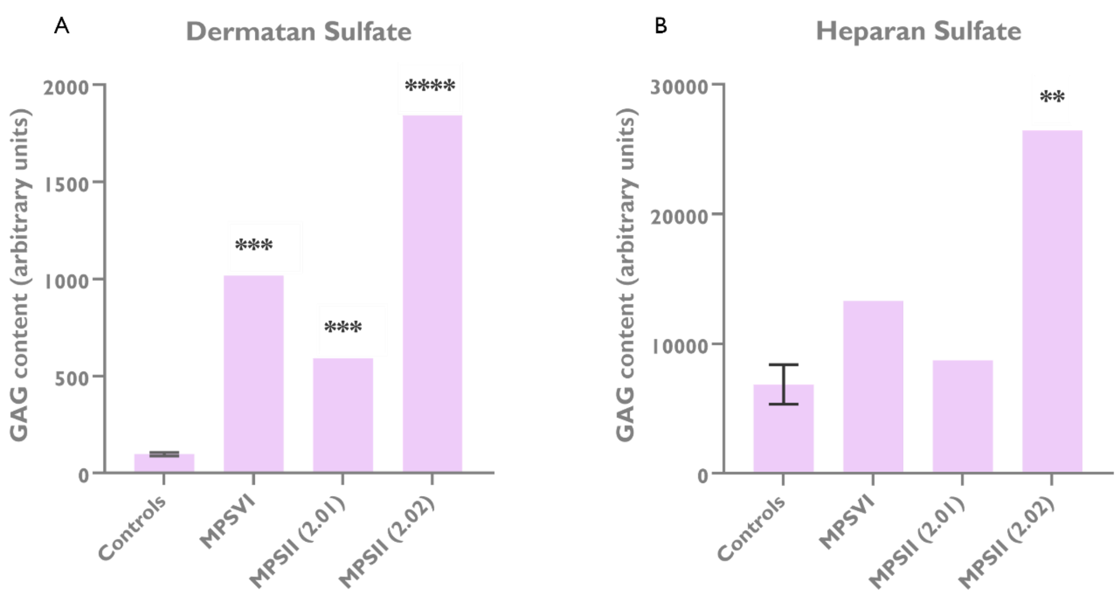



***Figure 21*** *- Measurement of GAG content in both WT and MPSs cell lines., with obvious increases of both substrates in disease cells: A-Heparan Sulfate; and B-Dermatan Sulfate. (**: p<0.05; ***: p<0.0005; ****: p<0.0001.)*

## 5.4. LAMP-1 staining

LAMP-1 staining was also performed, as yet another assessment of the subcellular LSD phenotype in these MPS-derived SHED cell lines. As referred in the *Introduction* section, LAMP-1 is one of the most abundant proteins in lysosomal membranes, and that is why its overexpression is usually related to lysosomal pathology. Again, the pathological phenotype was evident. In fact, when analyzing LAMP-1 staining results, a prominent fluorescence was perceptible in MPS VI and MPS II cell lines when compared with two independent controls (Figure 22).

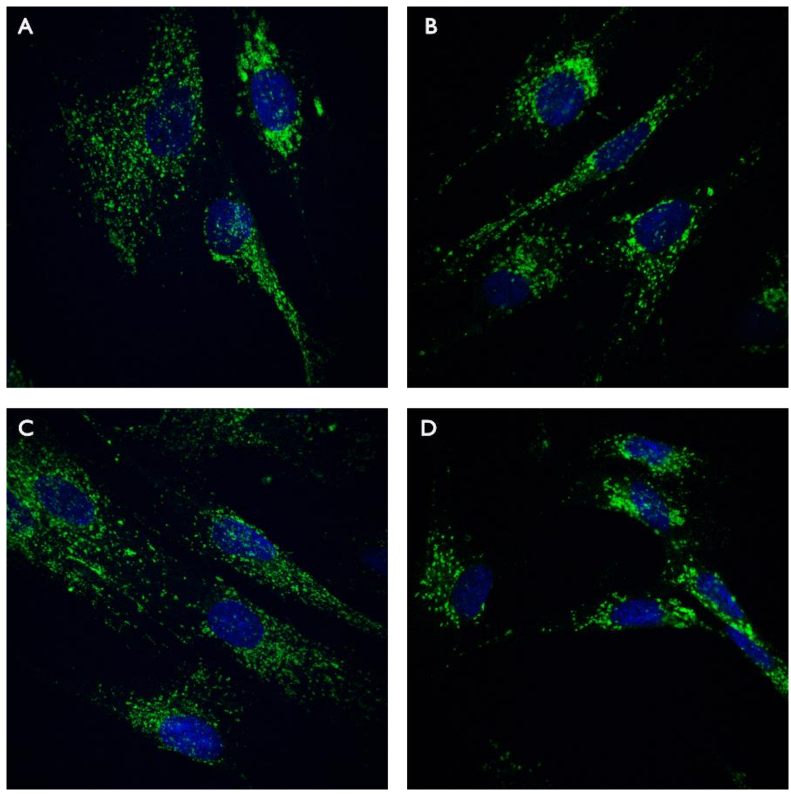



***Figure 22*** *- LAMP-1 staining. (A) and (C): immunostaining for control cell lines. (B): immunostaining for MPS II-derived SHEDs. (D) immunostaining for MPS VI-derived SHEDs. In both disease cases a prominent fluorescence is noticed, especially in the perinuclear space, when compared with control lines.*

# Discussion

## *Discussion*

The work summarized in this thesis was developed in a Research and Development group on Lysosomal Storage Disorders (LSD) that over the last years, has been focused on the design and pre-clinical evaluation of RNA-based therapies to correct or ameliorate the cellular phenotype of a few LSDs, including MPSs[3]. And while the theme for this thesis may seem somehow unrelated with that goal, truth is that those studies aiming at drug development were actually the trigger that led the team to reflect on the major obstacles, which may hinder their clinical translation. From that reflection, arose one of our current interests: the need for suitable disease models.

The main goal of this thesis was to develop an innovative cell model that could recapitulate disease-relevant features of MPSs, and to do it in a time- and cost-effective way.

In order to accomplish that goal, a naturally-occurring source of stem cells was chosen: the dental pulp. This tissue has been known for some time, to harbor a particular population of MSCs, which has been coined DPSCs. Quite remarkably, these cells are not only present in permanent teeth but also, in deciduous (baby) teeth. This means they can also be isolated from naturally exfoliated teeth, in a totally non-invasive way. Furthermore, taking into account that the most severe forms of MPSs are pediatric, i.e. have an early onset of symptoms, that sub-population of stem cells, the so-called Stem Cells from Human Exfoliated Deciduous Teeth, or SHEDs, seemed the perfect choice for this modeling purpose. Again, it is never much to stress that their collection causes virtually no pain or distress to the patients, since the teeth fall naturally, with no need for an active removal. And, even in the case of a surgical intervention being necessary, it is a minimally invasive procedure, which has absolutely nothing to do with the fact that those teeth will later be used for SHED cells' isolation. Those protocols are only performed when considered necessary for the patients' well- being, according to their responsible clinicians, dentists and/or orthopedists [242].

Overall, the establishment and detailed characterization of control- and MPS-derived SHED cell lines were an essential part of this work, which included not only a proper characterization of their MSC phenotype, but also a confirmation of their neural crest cell origin. Additionally, to make sure that these cells could actually recapitulate disease-relevant

[3] For an extensive overview on some of the RNA-based therapies our group has been developing for MPSs and their underlying rationale, see Annex 1, review paper 2: Santos et al., 2022 **Splicing Modulation as a Promising Therapeutic Strategy for Lysosomal Storage Disorders: The Mucopolysaccharidoses Example**.

features, the presence of three MPSs hallmarks was also addressed and confirmed: the underlying enzymatic activity deficiency; the consequent accumulation of GAGs; and, finally, the presence of an abnormal LAMP-1 staining pattern, which correlates with altered lysosomal positioning.

Altogether, these results will be discussed in further detail over the following sections.

## 1. Establishment of a protocol for primary cell culture of Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) *in house*

The overall success of the work described throughout this thesis relied, in the first place, on our team's access to a novel sort of sample, previously unstudied in the lab: deciduous teeth. Obtaining these samples depended on the support numerous healthy children and their families, who heard the call of our unexpected co-worker, "The 2020s Tooth Fairy", and accepted her plead to donate their recently exfoliated baby teeth.

Since the establishment of SHEDs had never been performed in our laboratory before, an intensive bibliographic search and selection of protocols to follow had also to be done. Overall, the basic protocol selection was quite straightforward. In fact, our prime intention was the generation of neuronal models from baby teeth, as we considered the neurological phenotype to be the most challenging for MPS diseases. Thus, we selected a published method, described by Goorha and Reiter [243] that described, with absolute detail, all the steps for the collection and transportation of teeth as well as extraction, passage, freezing, and thawing of stem cells from dental pulp.

However, after receiving and managing the first teeth, some adaptations were performed to either facilitate the whole technique, or make it slightly less expensive. In order to decrease its associated costs, we tried to change the *Transport Medium*. In fact, the original article stated this media should be constituted by *DMEM:F12 (50:50)* with *HEPES buffer* and 1% *Antibiotic-Antimycotic Solution*. The solution we optimized, on the other hand, used saline solution with the *Antibiotic-Antimycotic Solution* instead, and it worked similarly for the conservation purpose of the teeth. A few other changes were also performed, namely on the *DPSC Culture Medium* solution, on the different coating reagents used, and on the reagents used for Cell Detachment. Regarding the *DPSC Culture Medium*, we have not included the *Newborn Calf Serum* in the recipe. Thus, instead of having a final concentration of 30% serum (10% *Newborn Calf Serum* + 20% *Fetal Bovine Serum*, our media had only 20% *Fetal Bovine Serum* in total. According to our experience, one such percentage is enough to allow for rapid and effective cell proliferation, while it does not hold the same risk of culture contamination as a higher dose would naturally

imply. When it comes to the coating reagents, two different ones were successfully used for SHED cell cultures: *poly-D-lysine* (the one recommended in the original protocol) and *vitronectin* (the one standardly used in our laboratory for iPSC cell culture). Finally, we have also opted for a different, less expensive yet equally efficient cell detachment reagent: *Accutase*. Altogether, none of these changes had a negative impact on the success rate of the protocol, while strongly contributing to make it even more cost-effective.

In summary, during the course of this thesis, it was possible to implement *in house* this adapted version of the original protocol described by Goorha and Reiter [243] for the efficacious remote tooth collection and subsequent dental pulp extraction for growth and expansion of that particular subset of DMSCs, and applied it in dozens of controls with remarkable success. As originally reported by Goorha and Reiter, the process of growing these particular DMSC can take anywhere from 1 to 2 weeks and, at least in our hands, there seemed to be no particular correlation between the size of the pulp, or the time it takes to arrive at the lab (as long as the 48/72h interval is ensured) and the time it takes for the first cells/colonies to become visible in the plate. Overall, the whole method is extremely well-described in the publication we refer to, and according to our experience, it is not hard to implement in a lab with standard cell culture conditions.

There are, however, a few considerations we would like to make on the overall success of this cell culture protocol. While DPSC and SHED cell lines may be efficiently cryopreserved and thawed, their adherence rate after cryopreservation is far for optimal, at least in our hands. This seems to be in accordance with one of the troubleshooting comments of the original publication that refers to the "difficulty (of) growing DPSC lines from frozen stocks". On that section, the authors draw attention to the fact that it is very important to thaw these cells as fast as possible, when retrieving SHEDs from the liquid nitrogen cryobank. Thus, we always made sure to have the Falcon tubes already labelled and filled with pre-warmed *DPSC Culture Medium* (37°C) so that cells were immediately poured in it and the protocol was as fast as possible. Nevertheless, in our hands, there always seemed to be a tendency to get higher and faster cell adhesion rates when thawing cells from - 80°C, than when thawing them from liquid nitrogen storage, regardless of the passage we were recovering and the time they had been stored. As curious as this may seem we also saw that cells that had been kept for short periods in - 80°C, were usually faster to adhere than those which were stored in liquid nitrogen.

Therefore, while not initially envisaged, we are currently considering the possibility to establish a protocol for the immortalization of primary SHED cells, whenever we receive new

teeth from MPS patients. We believe that, by implementing one such protocol, we may not only increase cell viability after storage, but also decrease the time it takes for a culture to grow after freezing. Numerous protocols are currently known for cell immortalization and (at least) one of them has already been used in primary DPSCs by other authors [244], following a protocol described by Egbuniwe and co-workers, in 2011 [245]. Overall, it seems like a feasible approach, and that is why we are considering to attempt it in the future.

Another possibility we have been considering is that of using a higher concentration of serum, when freezing and thawing these cells. In fact, from our experience, the use of DPSC media with 20% *Fetal Bovine Serum* alone, does not seem to negatively influence the SHEDs' expansion rate when in culture. However, it is possible that, for longer storage periods, or to allow for a faster adherence and recovery after thawing, these cells may require a higher supplementation, thus benefiting from the use of 10% *Newborn Calf Serum* in the recovery medium.

Altogether, however, the success in implementing this whole protocol is undeniable, and its feasibility is remarkable.

To further validate the method, before requesting for patients' samples, we assessed, by qRT-PCR the expression of three consensus pluripotency markers in two randomly picked controls from all the established cell lines, and observed that both expressed all of them, as expected for any stem cell. Also noteworthy, we observed that the expression levels of those pluripotency markers increased over subsequent passages (from px2 to px7). That pattern was consistently observed for both cell lines, in all assessed passages and in three independent qRT-PCR assays. Thus, we hypothesize it may be associated with an increase of cell culture purity. This is consistent with the fact that some teams use fluorescence activated cell sorting (FACS) analysis to isolate DPSCs and/or SHEDs in the original culture and then stick with the selected ones for subsequent passages.

## 2. Establishment and Characterization of MPS-derived SHEDs

As soon as the whole method was successfully implemented and the presence of pluripotency markers confirmed in the established cell lines, the call for volunteers was extended to MPS patients and their families, under the terms and conditions described in the Materials and Methods chapter, section 1.1.1..

## 2.1. Characterization of the MSC phenotype

Whenever a novel MPS-derived cell culture was established and left to grow in a selective culture medium (here in termed *DPSC Culture Medium*), the first analysis that could be taken was the careful and systematic observation of the morphological aspects of these cells. In fact, in order to fulfill the minimal criteria to be classified as MSCs, they would have to, first and foremost, adhere to plastic. That was an obvious observation, since only those cells, which adhered to our *poly-D-lysine*- or *vitronectin*-coated plastic plates, were left to grow. Then, those adherent cells should show the so-called colony forming unit-fibroblast (CFU-F) [154,155], and that was precisely what we observed. At a strictly morphological level, all established cell lines presented with the expected morphology, characterized by a spindle shape, similar to that of fibroblasts.

But we wanted to study these cells in more detail, and perform a more accurate analysis, apart from the morphology observation and proliferation rate assessment. So, we expanded the catalogue of markers to quantify by qRT-PCR to include specific surface antigens whose presence (or absence, in a few cases) strongly correlates with MSCs identification. Obviously, we kept the pluripotency markers, already referred in the previous section, but then we added five other markers to the test: CD105, CD90, CD73, CD34 and MHCII.

Technically, MSCs are classified as multipotent stem cells and not as pluripotent stem cells. Still, as we have already seen, they do present a positive expression pattern of *OCT 3-4*, *Nanog*, and *Sox-2*, which are commonly used pluripotency markers [246–248]. DMSCs in general, and SHEDs in particular, are already known to express those markers for quite a while, now. In fact, that characteristic was already reported in healthy SHEDs back in 2009 in an original paper by Gronthos et al. [157], where their stemness character was confirmed. Nevertheless, the expression level of any of these markers, when compared with other commonly assessed MSCs markers is known to be weak. These data correlate nicely with our results, where all SHED cell lines presented with positive expression levels of these three pluripotency markers, but at a level, which was significantly lower than that seen for specific MSCs markers (CD105, CD90, and CD73). They also seem to be in accordance with what we saw on the iPSC cell line we used as a control: while positive, the levels of expression of *OCT 3-4*, *Nanog*, and *Sox-2* were much lower in the established multipotent SHED cell lines, than in the truly pluripotent iPSC line, which was triggered to overexpress those markers, through an artificial protocol. Interestingly, no studies comparing the expression levels of stemness markers between DMSCs and iPSCs are available, at least that we are aware of. Therefore, these results become even more interesting.

Additionally, specific MSCs markers were also measured, in both healthy and disease-derived SHEDs, as well as in that iPSC line (derived from Fabry fibroblasts) and, overall, the results were in line to what would be expected, according to the literature: MSC markers (*CD105*, *CD90*, and *CD73*) were the ones that displayed higher expression levels, thus supporting the MSC phenotype of the established SHEDs. The two other markers assessed, *CD34* and *MHCII*, are commonly described as absent in MSCs. They did, however, show a positive expression, even though with significantly lower levels than those observed for *CD105*, *CD90*, and *CD73*; they were actually comparable with the ΔCt value observed for the pluripotency markers. And, while this result seems unaligned with MSC requirements, as they are reported in the bibliography, when we look at individual papers where SHED and DPSC expression patterns for these markers were assessed, this observation is actually common. For example, recently, positive expression levels of MHCII were reported in a commercially available DPSC line, and considered a normal aspect [249]. Additionally, there is already literature commenting on the possibility that the absence of expression of those markers may not be mandatory for a cell to be classified as MSC, once several MSCs have been shown to express, at least to some extent, both of them [250,251].

Another characteristic that is strongly recommended to be evaluated in MSCs is the ability to undergo adipogenic, osteogenic, and chondrogenic differentiation, generating the respective cells. In this work, we performed all three differentiation protocols, using commercially available kits, which relied on the incubation of our original cell cultures with an appropriate induction medium for 21 days. By the end of that period, a morphological change was notorious in the cells submitted to osteogenic and chondrogenic differentiation. After adipogenesis, no obvious morphological changes were seen but, as already referred in the Results section 1.1. those morphological assessments were performed using a standard inverted light microscope. This means that, while drastic morphological changes such as those observed in the cells subjected to osteogenesis and chondrogenesis, may be easily documented, the same may not apply to those under an adipogenesis protocol because the morphological differences between SHEDs and adipocytes are much less obvious. So, it cannot be ruled out the possibility that, those same cells, observed by electron microscopy, for example, would appear significantly different. These results were also been described in 2003 by Miura et al. [164] and in 2014 by Chen et al. [252], when analyzing the multilineage potential of stem cells derived from dental pulp.

Still, we are currently working to improve our methods for multilineage cell detection. For chondrocytes and osteocytes, we are considering to take advantage of another protocol,

which is available in house for diagnostic purposes (total GAG quantification) to further validate the observed morphological chances. In fact, skeletal cells have been long known to present a prominent presence of GAGs [253–255]. Therefore, an alternative way to measure the efficiency of SHEDs' chondrogenic and osteogenic differentiation would be to analyze their total GAG content, and compare it to that of the same SHED culture before differentiation. Regarding SHEDs-derived adipogenic cells, the efficiency of differentiation may be assessed with the lipid Oil Red O (ORO) staining assays [249]. We do not have that protocol currently implemented in the lab, but it will be one of our goals in the near future.

### 2.2. Confirmation of the Neural Crest Cells Origin of Control and MPSs-derived SHEDs

An intrinsic aspect of stem cells from the oral cavity is their origin in neural crest cells. Thus, apart from the assessments we made to confirm the MSC phenotype of the established cell lines, we have also considered it relevant to evaluate whether they expressed common neural stem cell markers. Therefore, we performed a commercially available immunocytochemistry assay for four independent markers: Nestin, Sox-1, Sox-2, and Pax-6, and saw positive staining for all of them. Not surprisingly, the presence of Nestin and other neuronal precursor markers had already been described in the literature by several independent teams [163,256–259]. In general, Nestin, Sox-1, Sox-2, and Pax-6 are commonly assessed markers in neurodifferentiation protocols from iPSCs. These four neuronal markers are highly expressed in neural stem cells (NSCs) and have a key role in neuronal development. They are usually expressed in undifferentiated-CNS cells and downregulated over the subsequent differentiation into neural/glial cells [260–263]. This exact pattern was actually already observed in MPS-derived NSCs from MPSs I and II patients [139,141,152] Altogether, our results are totally aligned with what is published in the literature, further supporting not only the neural crest origin of these cells, but also their probable "neuronal fate", which ultimately may facilitate neurodifferentiation.

## 3. Modeling Mucopolysaccharidoses with SHEDs

As soon as our newly established MPS-derived SHED cell lines were analyzed for their stemness potential and their MSC phenotype, we moved on to analyze whether they were able to mimic the primary defect underlying the MPS phenotype in the patients from whom they were derived. So, a careful molecular characterization of their associated genotypes was performed, together with a quantification of each one's defective enzyme. Additional assessments to understand whether MPS-derived SHEDs were able to recapitulate other

disease-associated phenotypes, such as the presence of storage material and the abnormal distribution and/or quantity of lysosomes.

### 3.1. Molecular analyses and determination of Enzymatic activities

As referred before, it is a deficiency of different/specific lysosomal enzymes that lies in the beginning of the pathological events cascade of MPSs: the gene that encodes that particular enzyme harbors one (or two) mutation(s), which eventually prevents the enzyme's to complete its function. Consequently, the substrate it would degrade starts to accumulate giving rise to the pathology. Thus, if our goal was to develop an *in vitro* MPS model, that enzymatic defect had to be confirmed, both at molecular (associated gene mutation) and biochemical level (defective enzyme activity levels).

At a molecular level, the mutations present in each one of the MPSs-derived SHED cell lines were the following:

a) MPS II (Case 2.01): a previously reported rearrangement involving recombination between intron 7 of the *IDS* gene and sequences located distal of exon 3 in the *IDS* pseudogene (*IDS-2*) - GAATC>AGAGG (*IDSP1> IDS*) [227,234,238]. As expected for an X-linked disorder, this pathogenic variant was present in hemizygosity.
b) MPS II (Case 2.02): a hemizygous nonsense mutation (c.22C>T; p.R8*) mutation, previously reported in MPS II patients from other European populations [236,238,239].
c) MPS VI (Case 6.01): a single missense mutation (c.971G>T; p.G324V), present in homozygosity, previously known to correlate with a severe form of the disease [240,241].

Concerning enzymatic activity, as expected, when we compared the levels of arylsulfatase B activity in MPS VI-derived SHED homogenates with those observed in controls, a significant decrease was perceptible in the patient-derived sample.

For MPS II SHED homogenates, on the other hand, our results were even more obvious, with both patient-derived samples presenting with a total absence of iduronate-2-sulfatase activity. Altogether, these results were totally aligned with our expectations, as they validated the MPS VI and MPS II defects, both at molecular and biochemical levels.

Additionally, we have also measured the activity of several lysosomal enzymes not directly involved in MPS II or VI pathologies both in WT and MPS SHED cell lines. Not surprisingly, there weren't any significant differences between the results for controls and disease SHEDs for those enzymes. Together, these results further support our assumption that the methods,

which are implemented *in house* for LSDs diagnoses by enzyme activity measurements in leukocytes, can work in SHED cell homogenates as efficiently as they do in fibroblasts. Furthermore, and even though that data is not presented in this thesis, the registered enzyme activity values were in agreement with the standard values used to confirm the diagnosis in disease individuals in "*Unidade de Rastreio Neonatal- Departamento de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge*" (*data not shown*).

### 3.2. Glycosaminoglycans accumulation

Having confirmed the underlying enzymatic defects in our models, our immediate goal was to check whether those cells displayed the storage phenotype, which is the major hallmark of these disorders. In fact, GAGs' accumulation is the first consequence of the enzyme deficiency in MPSs. Therefore, it reveals an important pathological aspect to assess when it comes to the development of a disease model.

In order to quantify that storage, we used an extremely sensitive method: LC-MS/MS. With it, we were able to quantify, in the same run, two independent GAGs: DS and HS. By adding both, we could get an insight on the level of GAGs accumulation in each sample (SHED cell homogenates). Again, our results showed an increase in GAGs' content in MPS SHED samples, when compared with control-derived SHED homogenates. As referred in the Introduction section, DS storage is a hallmark feature of both MPS II and VI. Additionally, in neuronopathic forms of MPS II, the HS accumulation is also commonly observed in a higher level. Regarding MPS VI, while HS storage has not always been considered an expectable finding in this disorder, numerous teams have already reported that the accumulation of this substrate may also happen. Apparently, when highly sensitive methods are used to measure HS, this accumulation becomes far more evident. For example, when Tomatsu and collaborators measured plasma HS levels by ELISA in a panoply of MPS disease patients, those levels were shown to be altered in many more MPS than previously expected, taking into account what was known about the HS metabolic pathway. For example, plasma HS levels in all five MPS VI and 15% of MPS IV patients analyzed in that paper were elevated above the mean +2SD of the controls [264]. Overall, our observations are in total agreement with what is known about the pathology itself, but also with observation in previously existing models [139,220,254,255,265–268].

Notably, DS levels showed a significant difference in all of the MPSs SHEDS (with significant values in MPSII case 2.02) which is in line with clinical symptoms, confirming the multisystemic phenotype character. Regarding HS levels, in particular, even though an increased content was observed in all diseased SHEDs, that value was only statistically significant in case 2.02 (MPS II).

This observation is particularly relevant since numerous studies in LSD-derived iPSCs failed to reproduce the storage phenotype in the non-differentiated iPSC models.

### 3.3. LAMP-1 staining

Finally, we assessed yet another LSD-related subcellular phenotype. One of the most commonly reported pathophysiological aspects associated with MPSs is the increase/accumulation of lysosomes, which strongly correlates with either an increase in the LAMP-1 signal (a well-known lysosome membrane protein), or their abnormal subcellular localization, which correlates with an altered staining pattern [269]. In our MPS SHED cell lines, that altered staining pattern was obvious. In fact, it was possible to verify a strong perinuclear fluorescent LAMP-1 staining, which contrasted with the typical punctate subcellular localization (all over the cytoplasm) that was seen in healthy cells. Although we have not performed a quantitative analysis of the LAMP-1 signal, the abnormal staining pattern was evident. a proper signal quantification was not done, but, with only optic analysis, The identification of this feature was also seen in some MPS-derived iPSCs models [128] [147,148,152],

Altogether, the three tested hallmarks of MPSs are intrinsically connected: the enzymatic deficiency causes a non-degradation of its respective substrates, in this case, HS and DS. In turn, the accumulation of those GAGs within the lysosomal compartment could promote both organelle enlargement and an increased lysosomal biogenesis or decreased turnover, which eventually results in a higher number of lysosomes per cell. These events lead to a subsequent increase of lysosomes, which correlates with that of associated-lysosomal proteins, such as LAMP-1, the one we assessed in this work.

## 4. The knowledge acquired throughout this work

### 4.1. Comparison between the obtained results for SHEDs and the ones reported in literature for iPSCs

Overall, this is an extremely relevant study, as it allowed not only to implement a time- and cost-effective method to model MPS diseases *in house*, but also to show that the models established with it may actually circumvent some of the major drawbacks of iPSC technology. In fact, there are many studies, which indicate that iPSC may not accurately represent changes associated with neurological pathogenesis because they maintain residual epigenetic marks associated with their original cell type. Ultimately, this can lead to inappropriate gene expression in the newly derived iPSC neurons – or, at the very least, to an expression that is

not representative of what happens in vivo [270]. As many other authors have already discussed before us, this residual epigenetic signature, along with genomic instability [271], tumorigenic potential [272], and a high mutational load [273] raises concerns for the use of iPSC to model neurogenic disorders [219]. This is particularly obvious for disorders such as Alzheimer's or Parkinson's that often have complicated genetic and epigenetic etiologies which can alter the molecular changes indicative of the particular disease, but it cannot be ignored for monogenic diseases such as the ones analyzed in this thesis. Having a neural crest origin, our SHED-based cell models nicely avoid this issue. Furthermore, it was possible to verify that all major subcellular disease hallmarks are already detectable in SHED models. The same, however, does not happen with iPSC-derived MPS cell models. The storage phenotype, for example, is usually not visible in iPSC; instead, it is only evident after those cells are subjected to a differentiation protocol. A remarkable example is that of a 2019's work of Kobolák et al. [219] on MPS II, where the authors established 3 different MPS II-patient derived iPSC lines and differentiated them all to neuronal lineage. The panel of techniques they used to analyze them was immense, and allowed for insights not only on several pathophysiological features, but also on their possible origin. And, while summarizing does results goes far beyond the scope of this discussion, there are two particular results we would like to highlight: 1) some of the most significant disease features (elevated level of lysosomal marker LAMP-2 and intracellular GAG accumulation) were only visible in NPCs and totally mature neurons – never in iPSCs – and, 2) NPC cultures showed more similarities with disease-associated parameters than mature neuronal cultures, despite sharing the same genetic backgrounds. These observations lead the authors to conclude that NPC cultures *per se* may provide a good model system for the examination of basic cytopathological events in MPS II, without further differentiation into mature neurons or glia cells [219]. Importantly, when the same cytopathological features were assessed in our MPS II-derived SHED cell lines, a similar pattern was observed, further reinforcing not only the assumption that DPSCs are, in fact, naturally occurring NPCs, but also that they represent an easily accessible cell type, to address disease-relevant features, which are not always obvious, neither in patient-derived fibroblasts, nor in patient-derived iPSCs.

### 4.2. Other naturally-occurring sources of MSCs to model MPSs

As a final remark, it is also important to stress that the overall success of this work relied on a number of factors, some of which were absolutely beyond the control of the investigation, with the most obvious being our access to MPS children's baby teeth right after they felt. And, while many patient families have approached us and showed a remarkable interest in the

project, some of the affected had already lost all their deciduous teeth or, alternatively, had not yet reached the age where teeth usually start to fall. Still, it was possible to establish three-independent MPS cell lines, representative of two different diseases and three different genotypes. Nevertheless, some of this technique's drawbacks, namely the small time-window in which deciduous teeth samples are available for each patient, and the difficulty to access them in the first 48h since it falls, have led the team to consider other possible alternatives. Thus, we are currently considering the possibility of using other naturally-occurring sources of MSCs, apart from the dental pulp, to establish cell cultures that may be of use for MPS disease modeling *in vitro*: urine. In fact, human urine-derived stem cells (USCs) are another type of MSCs with proliferation and multi-potent differentiation potential that can be readily obtained from voided urine using a non-invasive protocol and with minimum ethical restrictions. These cells express surface markers of MSCs, but not of hematopoietic stem cells, express the stemness-related genes *NANOG* and *Oct 3-4* and show telomerase activity [223,224,274,275]. When cultured in appropriate media, USCs may differentiate into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic, and neurogenic lineages. Thus, USCs are yet another alternative source of SCs that can be used as a valuable *in vitro* model to study genetic diseases, with potential applications in regenerative medicine, cell therapy, diagnostic testing and drug screening [225].

Yet another possibility, which is currently being considered is the use of adult human third molar teeth, from where we may also isolate dental pulp stem cells. While there are slight variations in the protocols described in the literature for the isolation of DPSC from this source, the overall methods is not significantly different from the one we have already implemented *in house* for SHED cell culture. This type of sample would allow us to significantly increase the number of eligible patients', because our recruitment platform would be much larger than the current one: it would move from children who are currently losing their baby teeth, to virtually any patient, regardless of his/her age. Again, we won't be asking for an active removal of third molar teeth; only the individuals, who need to get them removed for medical reasons will qualify. This may seem a slight change, but the fact is that the surgical removal of human third molars, also known as wisdom teeth, is quite a common procedure. These teeth grow in the back of our gums, and are the last teeth to come through, usually during the late teens or early twenties. By that time, all the other 28 permanent teeth are usually in place, so there isn't always enough room for third molars to grow properly. Wisdom teeth that grow like this are known in dentistry as impacted, and their surgical removal is the most common surgical procedure in the orthodontist field. This picture is probably even more prominent in

individuals who suffer from MPS, particularly from the forms, which most severely affect the skeletal system. In fact, amongst some of the most common and obvious orofacial abnormalities in MPS patients, are maxillomandibular abnormalities. GAG accumulation in soft tissues, cartilage, and bones and secondary cellular responses to accumulated GAGs are probably the culprit to abnormalities in orofacial soft tissues, orofacial bones, and teeth [276]. That is why MPS patients are frequently subjected to teeth removal surgeries, among other orofacial interventions, and that is also why we believe it makes sense to include surgically extracted permanent teeth in this study.

## 4.3. Beyond science

There is yet one final issue we would like to discuss, even though it may seem to fall slightly out of the scope of this thesis. It is the opportunity this work gave us, not only to contact with patients' associations and families, but also to approach the so-called 'general public'. And that is something that constitutes a science of its own: the so-called Science Communication. So, we took advantage of some of its most well-known tools, to address families, and ask for their help to implement these protocols: plain language, and storytelling. And we wrote a text, a call for volunteers, which relied almost exclusively on them. Stories are a great tool for public engagement. So, we wrote a simple story, in a simple way: we created a new character inspired by the Tooth fairy Story, the "2020 Tooth Fairy". This "new tooth fairy" will not leave a penny for a tooth. But she leaves hope under the child pillow. That tooth could be the hope to find a cure for this disease and save other children's lives. With this simple "science tale", we found a way to engage families in our scientific work, triggering curiosity and awe.

While the text was initially written to ask for a dozen volunteers, it actually granted the attention of thousands of families, who were actively willing to participate. And, while it would not be feasible to enroll them all, we did extend the study to almost 50 families. Ultimately, that success showed us how effective and engaging science communication can be, while drawing our attention to the fact that people may actually be willing to know more about these rare genetic diseases, as strange and uncommon as they may seem. While it may not be that obvious, rare diseases are a major public health issue. They are also the most impressive way of showing how genetics work and how vital and finely regulated is every single metabolic pathway. Therefore, educating society on their existence, their intrinsic causes and the hurdles they pose to affected individuals and their families, should not be neglected.

# Conclusion and

# Future Perspetives

## *Conclusion and Future Perspectives*

This work was aimed to develop new cell models that could accurately recapitulate disease-relevant features of MPSs, in a time- and cost-effective way. Overall, the work we here described can be divided into three major parts: the establishment of the method to successfully collect and isolate SHEDs *in house*; the subsequent characterization of not only controls but also patient-derived SHED cell lines and the assessment of their potential to accurately model MPSs.

In general, the implementation of the whole protocol in the laboratory, and the establishment of SHED cell lines was successfully achieved. In fact, we managed to establish over 30 independent control SHED cell lines, and three MPS-derived SHEDs cell lines: two from unrelated MPS II patients, and one derived from an MPS VI patient). Overall, this remarkable number of controls allowed us to assess, with a considerable degree of certainty the normal range of activity levels of several lysosomal hydrolases, further validating the assumption that most of the methods used *in house* for diagnostic purposes, would easily apply to those cells, providing similar results. Furthermore, it will also allow us for age- and sex-matches with virtually any patient sample we get, thus significantly reducing the variability that could arise from patient vs controls' comparisons not taking these factors into account. This will be particularly relevant to ensure robust conclusions and more accurate/adequate genotype-phenotype correlations.

Then, an exhaustive molecular and biochemical characterization, where it was possible to confirm the pluripotency status, and the MSC phenotpype of both control- and MPSs-derived SHEDs, by qRT-PCR analysis of specific transcription factors and/or specific cell surface antigens was performed. Also achieved, with a significant degree of success, was their subsequent multilineage differentiation, with visible changes in chondrocytes and osteocytes cells. Additionally, a first attempt to generate neural cells was also accomplished, even though the generated cells could only be analyzed at a morphological level. Still in the SHEDs characterization studies, another important result was the unequivocal confirmation of their neural crest cells' origin by immunocytochemistry.

Having clearly demonstrated and extensively characterized the established SHED cell lines, we then focused our attentions/efforts in understanding whether the major MPS disease hallmarks where present in our patient-derived SHED cell lines. After a series of standard protocols, it was possible to clearly demonstrate their underlying enzyme deficiencies, both at the molecular and biochemical levels. These first results were not surprising, since our models

are patient-derived and there is no report so far on any LSD-derived cell line that does not recapitulate the genetic defect or the enzyme activity deficiency that the patient from whom it was isolated harbours. However, the same is not true for many cytopathological disease hallmarks, such as primary and secondary storage, or abnormal lysosomal staining. All these features are frequently absent in a number of patient-derived cells, or artificially generate models. And here, our newly-developed models had a completely different result: we could prove beyond any doubt that these cells present, already in a pluripotency stage, well-known hallmarks of disorder including a pronounced GAGs accumulation and an aberrant pattern of LAMP-1, when compared with control SHEDs, thus qualifying as disease-relevant models.

Overall, this work represents an absolute innovation in the field, as it is the first time stem cells from the oral cavity are ever isolated from an LSD patient, and on top of that studied for their modeling potential. In fact, to the best of our knowledge never before had LSD patient-derived SHEDs been either established or used for differentiation into specific cell types.

It is also worth mentioning that LSD modeling is quite a recent line of research in our lab. Therefore, many of the protocols here described were either implemented for the first time during this thesis, or adapted to apply to this novel type of sample. And, given the positive results we got on the modeling potential of SHEDs, plenty of future perspectives may be envisaged, many of which will actually be performed in the near future, as the lab has been granted funding to pursue this ongoing work.

In summary, regarding the establishment and characterization of MPS-derived SHED cell lines, our goal is to:

- Increase the number of patient-derived SHED cell lines and, ideally, expand the catalogue of MPSs available in the lab.

- Enlarge our patient recruitment platform, by adapting our currently implemented protocol to accommodate yet another sort of patient sample: adult human third molar teeth, from where we may also isolate dental pulp stem cells. Additionally we intend to take advantage of urine as an alternative source of stem cells,where a higher number of samples and an easier collection method is achieved.

- Perform a qualitative analysis for adipogenic, chondrogenic and osteogenic differentiation, through ORO staining, GAGs content, respectively.

- Perform a quantitative analysis, through qRT-PCR of specific markers for adipogenic (e.g.: *Peroxisome proliferator- activated receptor gamma (PPARG)*

*and Lipoprotein Lipase* (*LPL*) expression levels), chondrogenic (e.g.: *Collagen type II alfa-I* (*COL2AI*) and *Aggrecan* (*ACAN*) expression levels) osteogenic (e.g.: *Collagen type I* (*COL1*) and *Osteonectin* (*ON*) expression levels).

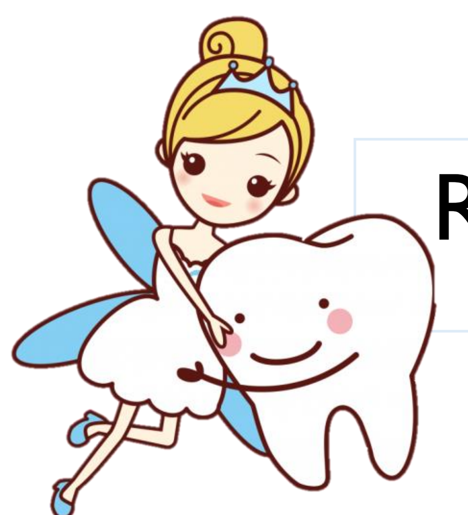
For modeling purposes, our aim is to:

- Perform a quantitative analysis of LAMP-1 staining in MPSs-derived SHEDs compared with healthy-derived SHEDs, to further validate the pattern we have already observed;
- Optimize the neurogenesis protocol by selecting different neuronal induction media and/or individual supplements, and evaluating each one's potential to promote a fast and effective differentiation of this particular type of MSCs. As soon as we succeed, the resulting totally differentiated neurons and/or astrocytes will also be extensively characterized.
- Repeat the subcellular phenotype assessments we have performed in patient-derived SHEDs in terminally differentiated osteocytes, chondrocytes and neurons/astrocytes to assess whether any significant differences are observed and conclude which type of cell has the greatest modeling potential.
- Perform additional studies in our cells models to unveil the different pathophysiological events, which may be involved in MPS pathology (e.g.: Caspase 3/7 assay and XBP1 assay).

Altogether, our search for MPS patients' teeth or even for deciduous teeth from patients suffering from other LSDs is still ongoing, and there will be always space in our lab to establish a novel patient-derived cell line whenever needed, as well as to perform its molecular and biochemical characterization in a proper way.

Indeed, we strongly believe this non-invasive method will become routine not only in our lab but also in many cell culture labs as it may provide new clues about a group of disorders that are yet, very much unexplained. In the particular case of our lab, those cells will also constitute an optimal platform for drug testing *in house*.

Also noteworthy, our models will be published as lab resources and made available for the whole LSD community.



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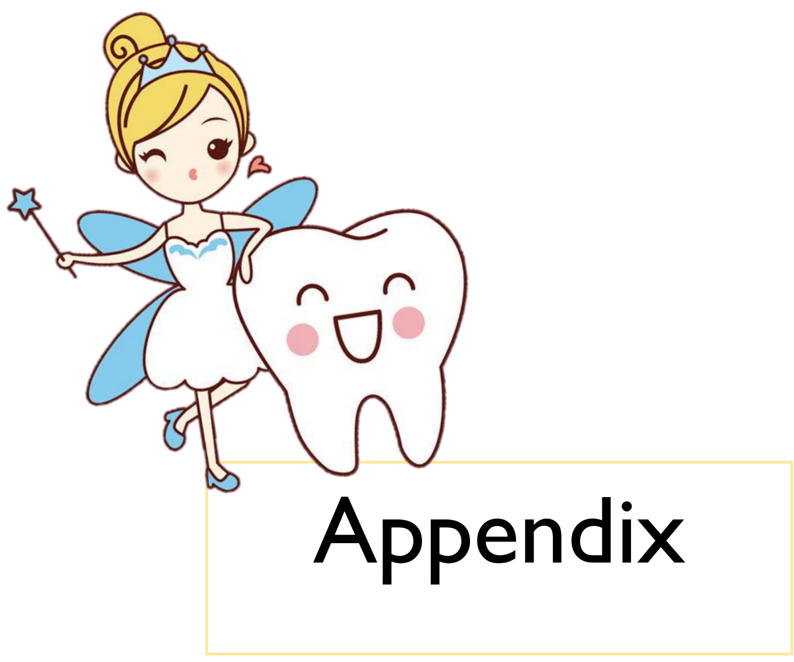

# Appendix

# Annex I

- Review Paper 1: Carvalho et al., **Neurological disease modeling using Pluripotent and Multipotent Stem Cells: a key step towards understanding and treating Mucopolysaccharidoses** [Under Preparation]

- Review Paper 2: Santos et al., 2022 **Splicing Modulation as a Promising Therapeutic Strategy for Lysosomal Storage Disorders: The Mucopolysaccharidoses Example**

*Review*

# Neurological disease modeling using Pluripotent and Multipotent Stem Cells: a key step towards understanding and treating Mucopolysaccharidoses

[*under preparation*]

**Abstract:** Despite extensive research, the links between the accumulation of glycosaminoglycans (GAGs) and the clinical features seen in patients suffering from various forms of Mucopolysaccharidoses (MPSs) have yet to be further elucidated. This is particularly true for the neuropathology of these disorders, even though the neurological symptoms are currently incurable, even in the cases where a disease-specific therapeutic approach does exist. One of the best ways to get insights on the molecular mechanisms driving that pathogenesis is the analysis of patient-derived cells. Yet, not every patient-derived cell holds potential to recapitulate relevant disease features. For the neurodegenerative forms of these diseases in particular, it is challenging to grow neuronal cultures that accurately represent them because of the obvious inability to access live neurons. This scenario changed significantly since Yamanaka et al. published their protocol for induction of pluripotent stem cells (SC) from adult human fibroblasts. From then on, a series of differentiation protocols to generate neurons from induced pluripotent stem cells (iPSC) was developed and extensively used for disease modeling. Currently, human iPSC and iPSC-derived cell models have been generated for several MPS and numerous lessons were learnt from their analysis. Here we review most of those studies, not only listing the currently available MPS iPSC lines and their derived models, but also summarizing how they were generated and the major information different groups have gathered from their analysis. Finally, and taking into account that iPSC generation is a laborious/expensive protocol that holds significant limitations, we also comment on a tempting alternative to establish MPS patient-derived neuronal cells in a much more expedite way by taking advantage of the existence of a population of multipotent SC in human dental pulp, to establish mixed neuronal and glial cultures.

**Keywords:** Mucopolysaccharidoses; Disease Modeling; *in vitro* models; induced Pluripotent Stem Cells (iPSCs); Dental Pulp Stem Cells (DPSC)

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## 1. Introduction

Lysosomal storage disorders (LSD) are a group of rare diseases caused by mutations in genes that encode lysosomal enzymes, lysosomal membrane proteins or transporters and in a few cases by other cell proteins that are important for lysosomal function. This leads to an accumulation of undegraded substrates, which ultimately causes a broad range of highly debilitating clinical symptoms affecting multiple organs/systems, including the central nervous system (CNS) [1]. Among the LSDs that may present with severe neurological phenotypes, are Mucopolysaccharidoses (MPSs), which are caused by impaired degradation of glycosaminoglycans (GAGs), with consequent intralysosomal accumulation of undegraded products [2]. Quite remarkably, none of the available therapies for this sub-group of disorders works over the neurological symptoms. Instead, they are limited to treating non-neurological signs [3]. Thus, there is an urgent need for the development of new ones that can tackle the neuronal pathogenesis. A crucial step towards the development of those approaches is the existence of suitable disease models, which can be used to both further understand the pathophysiological mechanisms that underlie the phenotype and adequately test those therapeutic strategies *in vitro*. Here we will review some of those models and the major results that other groups have published on the pathophysiological mechanisms underlying this particular subset of LSDs. We will highlight the different patient samples they used to start with, and the protocols they relied on. Particular attention will be given to the induced Pluripotent Stem Cells (iPSCs) potential to mimic disease-relevant phenotypes and to the methods others have used to assess them. Finally, we will also mention a few studies, which have provided *in vitro* proof of principle on the potential of *ex vivo* genetically-corrected iPSC -derived cells for therapeutic purposes.

Overall, the results here reviewed strongly support the utility of iPSCs for the study of MPSs. Still, iPSCs generation is a laborious and expensive protocol. Furthermore, the use of iPSCs has a number of limitations, which should not be ignored. That is why in our lab we are addressing the question of whether alternative sources of stem cells (SC) may exist, holding a similar potential for disease modeling in these rare yet life-threatening genetic disorders. In fact, recent studies have shown that dental pulp provides a niche for diverse arrays of dental mesenchymal stem cells (MSCs), and they are now being established in our laboratory for the study of LSDs, particularly MPSs. This approach is non-invasive, cost-effective, and can be established in any laboratory with standard cell culture conditions. And as we will briefly highlight in this manuscript, it may provide another potentially effective approach for investigating cellular and gene expression changes that occur in monogenic diseases.

## 2. Lysosomal Storage Diseases

Lysosomes have in their composition around 60 acidic hydrolases responsible for the degradation of a variety of substrates including proteins, lipids, carbohydrates and nucleic acids [4,5]. When one or more lysosomal enzymes fails to fulfill its function, the substrate(s) it would degrade starts to accumulate in a process which, eventually will result in cellular toxicity and even cell death [6–8]. In general, those enzymatic dysfunctions have a genetic origin, as they are caused by mutations in any of the genes that encodes for the defective protein. This sort of monogenic disorders characterized by intralysosomal substrate accumulation constitutes a large group collectively known as LSDs [9]. This group comprehends around 70 disorders being almost all characterized by a recessive autosomal pattern of inheritance. Currently, only three exceptions are known, all of them X-linked.

Classically, LSDs are classified into different subgroups depending on the substrate that is accumulated [10]. According to that classification, we can distinguish five major groups

of LSDs: Sphingolipidoses (those which accumulate sphingolipids), Mucopolysaccharidoses (those which accumulate GAGs), Oligosaccharidoses (those which accumulate oligosaccharides), Sialic Acid disorders (those which accumulate sialic acid) and Mucolipidoses (which accumulate a number of different substrates, namely of mucopolysaccharides, sphingolipids and glycolipids). But not all LSDs fit into this traditional classification. That is why we can usually find (at least) two extra categories in most of the tables where these disorders are listed: the so-called Neuronal Ceroid Lipofuscinoses (NCLs) and a general category coined Miscellaneous (whose disorders may accumulate substrates as diverse as polysaccharides and amino acids) [11]. There is, however, an obvious link between the majority of the referred disorders: the neuronal storage of undegraded or partially degraded substances, with subsequent cell death in the brain. Accumulation within this system result into a panoply of symptoms including neurocognitive decline, blindness, seizures and, ultimately, premature death. Still, not every LSD shows a direct/obvious CNS involvement. Some LSDs present in a much more multisystemic way and, for some the milder forms may actually lack neurological symptoms. Symptoms like hepatosplenomegaly, cardiomyopathy, fibroelastosis, dysostosis multiplex and cervical spinal cord strangulation are often part of the LSD phenotype, and may be the only clinical manifestations in a number of patients [12]. In general, the clinical manifestations depend on the substrate accumulated and on the site where that accumulation occurs. Furthermore, depending on the specific function of the enzyme, which is either missing or dysfunctional, and on its level of deficiency, storage may accumulate at different rates, causing the disease progression to be significantly different [12].

Generically, LSDs are rare diseases. Nevertheless, when considered as a whole, their prevalence may be as high as 1 in 5,000 [10]. Depending on the group and/or subgroup of diseases, there are differences in the severity of symptoms, rate of progression, and organs/systems affected. Still, regardless of their overall severity, LSDs are characterized by a relentless progression of symptoms and no cure is yet known for any of these disorders. There are, however, four different approaches, which have been explored for a number of them and some of them have actually reached the clinic [13]: Enzyme Replacement Therapy (ERT) [13]; Hematopoietic Stem Cells Transplantation (HSCT) [13]; Substrate Reduction Therapy (SRT) [10,13] and Chaperone Therapy [13,14]. It should be noticed, however, that these therapies are only available for a restrict number of LSDs and, even in the cases where a therapeutic option is available, it may fail to address all of the disease's symptoms, as we will extensively discuss throughout this review.

The most widely used therapeutic approach in the field is also the first one to have been developed: ERT. Briefly, ERT relies on a very simple principle: if LSDs are caused by an enzyme deficiency, one may overcome them by simply giving the enzyme that is missing to the patients who suffer from its dysfunction. Easier said than done, but still, a number of recombinant enzymes are now available in the market and being used by different LSD patients worldwide [15]. Those ERT formulations are administrated intravenously in a periodic manner. Briefly, the recombinant enzyme gets internalized into the cells by the so-called mannose-6 phosphate receptors (M6PR), and reaches the lysosomes through the mannose-6-phosphate pathway, where it may fulfill its function. The existence of mannose-6 phosphate receptors within the plasma membrane also allows for subcellular cross correction. Meaning: the recombinant enzyme may move from one cell to the next one, thus maximizing its therapeutic effect [15]. However, ERT does hold a series of drawbacks, for instances it may lead to the production of antibodies against the synthetic enzyme. Furthermore, recombinant enzymes do not reach all organs/systems. For example, traditional ERT does not reach the CNS, thus being a real therapeutic option only for non-neurologic diseases or for their non-neurological forms. Despite their limitations, ERTs for Gaucher Disease [16], Fabry Disease [17], Acid Lipase Deficiency [18], Ceroid lipofuscinosis type 2 [19], Niemann-Pick diseases type C [20] , $\alpha$- Mannosidosis [21], and

MPS I, II, IV, VI, and VII [22] are, nowadays, a reality and numerous patients have benefited from them over the last decades. Additional clinical trials with novel enzymes and alternative delivery routes are also ongoing [23]. Overall, ERT is not a cure, but it does significantly increase enzyme activity in many disorders, thus improving their associated clinical symptoms [24].

Another therapeutic approach for LSDs, which has been around for a few decades now, with very good results for a few diseases is HSCT [25]. Briefly, we can distinguish 3 types of HSCT: allogenic (when the transplanted cells are derived from a healthy and fully-matched donor); syngeneic (when the transplanted cells are derived from an identical twin); and autologous (when the transplanted cells are derived from the patient before the procedure). While allogeneic HSCT is the standard of care these days for a few LSDs, either syngeneic or autologous transplants are virtually better options, as they work around some of the acute complications associated with HSCT such as veno-occlusive disease of the liver, acute and chronic graft *versus* host disease , and opportunistic infectious conditions. In those two cases, however, the cells which are collected need to be genetically modified *ex vivo* to a normal function. Currently, those approaches are under clinical trial for a few LSDs [26–30]. Regardless of the HSCT type, in terms of procedure, its principle is simple: first, the patient needs to receive some type of therapy that will inhibit the immune system (to prevent rejection); then the modified cells are injected in the patient. Due to their stemness potential, the graft cells, which are capable of synthesizing functional target enzymes, will rapidly proliferate and differentiate providing a natural, endogenous source of the enzyme, which was previously missing [31].

Still, this approach does not seem to be effective for a number of LSDs where, in theory, it should work [32]. There are, however, a few diseases for which this procedure is highly recommended and does show exceptional results if performed soon enough. That is the case of one particular form of MPS: the Hurler syndrome (the severe forms of MPS I). Transplantation is still considered the "standard of care" for patients suffering from that syndrome. Nevertheless, this procedure in only effective when performed at the very initial stages of the disorder. In fact, it has only been shown to enhance the cognitive function in patients with less than 9 months [9,10,25]. Even though Hurler seems to be the perfect example on the success of HSCT, there are some general considerations we can draw for other LSDs to which may apply. Usually, visceral symptoms can be improved, whereas skeletal lesions remain relatively unaffected. The effect on neurologic symptoms varies. Still, HSCT remains a viable treatment option in those LSDs where data supportive of disease stabilization or amelioration is known (reviewed in [33]).

But there are two other, more recent approaches, which may be used to overcome the LSD-associated pathology. The first one is SRT, with licensed products available for Gaucher Disease and Niemann-Pick Type C. Again, its rationale is quite straightforward: it promotes an overall reduction of the accumulated substrate(s) by inhibiting its biosynthesis, thus ameliorating the associated phenotype(s). Unlike ERT, the presently available substrate reduction drugs are orally administrated, and some of them have the ability to cross Blood-Brain Barrier (BBB) achieving an effect on CNS [34]. Still, this option has a slower onset efficacy, and so far, it is restricted to sphingolipidoses. The conjugation of SRT with other therapies may significantly improve the treatment of LSDs [10,34].

Finally, there is also the so-called chaperone therapy. Pharmacological chaperones are small molecules defined by their ability to help a protein to fold correctly [35]. By doing so, those molecules will help their target protein escape proteasomal degradation and reach an adequate subcellular destination, where it can exert its function. Basically, this molecule binds to the misfolded protein in the endoplasmic reticulum (ER) forming a stable complex that prevents the misfolding. When the complex arrives to the lysosome,

dissociation occurs. As a result, a functional or partially functional protein gets internalized into that organelle, where it can exert its activity [14]. It is worth mentioning that this sort of therapeutic approach may only work for disease-causing missense mutations. So far, Fabry disease (one of the most common LSDs worldwide) is the only LSD with an approved chaperone therapy: migalastat (Galafold®, Amicus Therapeutics). This drug is currently being used in the clinic for a significant number of Fabry disease patients, all harboring missense mutations that cause misfolding of α-galactosidase, and has been shown to improve the associated cardiac and renal symptoms [36,37]. And, while no other chaperone molecule has reached the clinic so far, several studies are being performed in other LSDs [38–42].

## 3. Mucopolysaccharidoses

Among the LSDs in need for better and more effective therapeutic options are the Mucopolysaccharidoses (MPSs). The MPSs subgroup includes seven different disease types, all of them accumulating glycosaminoglycans (or GAGs) as the primary substrate. An overview of each individual disorder is described below.

MPS I is one of the most common forms of MPS and the first MPS type treated with ERT (available since 2003) [43]. At a clinical level, MPS I may be divided into three subtypes: Hurler (OMIM #607014), Hurler-Scheie (OMIM #607015), and Scheie (OMIM #607016) depending on the disease severity [44]. Hurler syndrome is the most severe form of them all and Scheie is the mildest, with Hurler/Scheie being somehow intermediate phenotype but in general, type I has an incidence of 0,11 [45] to 3,62 [46] per 100.000 live births (reviewed in [47]). As the majority of LSDs, MPS I is characterized by a progressive pattern that includes several stages of clinical manifestations. In this multisystemic disease during the first 6 months of life, the children present symptoms such as coarse facies hepatosplenomegaly, and upper airway obstructions that usually evolve to more specific and severe symptoms associated to a constant increase in the accumulation of GAGs in the soft tissues, bones, spleen and liver. Overall, dysostosis multiplex is considered the most common clinical symptom of MPS I [48]. Regardless of the clinical presentation, *IDUA* is the affected gene in this disorder. Mutations in this gene, which encodes for α-L-iduronidase (IDUA; EC 3.2.1.76), lead to an enzyme deficiency that ultimately results in heparan and dermatan sulfate (HS and DS, respectively) accumulation [49]. To date, 359 ([50]) mutations are identified for this gene [51], and currently, there are two possible therapeutic options: ERT and HSCT, which is only used in the most severe form of the disease and, preferably in the first years of life [52]. Regarding ERT, there is only one recombinant enzyme approved for MPS I: laronidase (Aldurazyme®, Genzyme). As every other ERT, this recombinant enzyme is injected into the blood circulation, which leads to the correction of the enzyme deficiency in various organs and tissues, except the brain, once it does not cross the BBB [53,54].

MPS II (OMIM #309900), or Hunter syndrome, is the only X-linked MPS disease; all the other MPSs are autosomal. Thus, in the Hunter syndrome, males are the most affected, with a prevalence of 0,1 [55] to 2,16 [56] in 100.000 live births (reviewed in [47]). Two forms of the disease may be distinguished: neuronopathic and non-neuronopathic, being the most severe the CNS-associated [57]. Regarding clinical manifestations, the skeletal, cardiac and respiratory systems are the ones mostly affected. In the most severe cases, adding up to the symptoms affecting the previously referred systems, there is also an involvement of the CNS. Usually, for the neuronopathic form, the average life expectancy is around 10-15 years of age, while the individuals who suffer from the attenuated one may live beyond 50 years [58]. Regardless of the subtype, MPS II is caused by mutations in the *IDS* gene, which encondes the enzyme iduronate 2-sulfatase (IDS; EC 3.1.6.13). The *IDS* gene is split into 9 exons, spanning approximately 24 kb [59]. There are around 817 mutations

identified to date, which may cause this syndrome ([50]). The iduronate 2-sulfatase deficiency leads to the accumulation of two substrates: HS and DS. Regarding MPS II therapeutics, ERT with idursulfase (Elaprase®, Shire) is the first choice for patients with this condition [60].

MPS type III, also known as Sanfilippo syndrome, may be subdivided into 4 subtypes: IIIA (OMIM #252900), IIIB (OMIM #252920), IIIC (OMIM #252930), and IIID (OMIM #252940). Each particular subtype is associated to a unique enzymatic defect: MPS IIIA is caused by the deficiency of the enzyme Heparan-*N*-sulfatase (SGSH, EC 3.10.1.1); MPS IIIB, by its turns is caused by defects in the enzyme *N*-acetylglucosaminidase (NAGLU, EC 3.2.1.50); in MPS IIIC the protein involved is the transmembrane enzyme, acetyl-CoA:Glucosamine *N*-acetyltransferase (HGSNAT, EC 2.3.1.78) and, finally, the MPS IIID is caused by defects in *N*-acetyl-glucosamine-6-sulfatase (GNS, EC 3.1.6.14). Regardless of the enzymatic defect itself, all of them are associated with a severe deterioration of neurological function [61], which results in a number of clinical symptoms either directly or indirectly related to a CNS dysfunction, such as behavior problems, sleep disturbances, hearing impairment, development regression, recurrent infections in the respiratory tract, and facial dysmorphology [62,63]. The general prevalence is 0,06 [64] to 1,89 [65] in 100.000 live births (reviewed in [47]), with subtypes A and B being more common for most populations than C and D [66]. Regardless of the affected genes, the stored substrate is always HS.

Various mutations were already identified for the different forms of MPS III [67]: in the case of *SGSH* gene (with a total of 8 exons and associated with subtype IIIA), 163 mutations have already been identified; in subtype IIIB, 215 mutations have already been identified in any of the 6 exons that constitute the *NAGLU* gene, or their surrounding intronic sequences; in the *HGSNAT* gene, around 93 mutations along the 18 exons and their respective introns are known to cause the deficiency observed in subtype IIIC. Finally, in subtype IIID, where the *GNS* gene (which spans 14 exons) is mutated, only 25 mutations were identified [50]. Unfortunately, there is no approved treatment for these neurologic diseases. On the one hand, while it has already been attempted by several different teams, HSCT has proven virtually no benefit over the neurocognitive sympyoms [68–72]. On the other hand, ERT is hard to apply, once classically formulated enzymes do not penetrate the CNS. Moreover, in the case of MPS IIIC, for example, ERT is not an option, once the deficient enzyme is a transmembrane protein.

There are, however teams attempting brain-specific delivery of both ERT and chemical compounds for MPS type III. In general, there are three strategies to increase the delivery (reviewed in [73]): enzymatic modulation, route(s) of administration [74–76], and increase of enzyme dosage. In addition, cellular and genetic therapies represent approaches that have gained importance when it comes to BBB delivery (reviewed in [77]). Targeting brain cells through enzymatic modulation consists of the combination of the enzyme with protein/peptides than can facilitate BBB crossing (reviewed in [78,79]). In the cellular and genetic therapies field, among other possibilities, gene therapy with the use of adeno-associated virus has been stealing a lot of attention with extensive works to reach the BBB and have the intended effect [75,80–82]. Besides the modifications above referred, substrate reduction therapy (SRT) constitutes also an alternative to get the BBB [83–85]. The development of a valuable treatment has reached very high levels of need so that regulatory initiatives to support the development of a possible treatment are commonly found [61,67,86,87].

There are two different forms of MPS IV, each one caused by a single enzymatic defect: *N*-acetyl-galactosamine-6-sulfatase (GALNS; EC 3.1.6.4) deficiency underlies MPS IVA (OMIM #253000) while β-galactosidase (EC 3.2.1.23) defects cause MPS IVB (OMIM

#253010). The involved genes are *GALNS* and *GLB1*, respectively. MPS IV, or Morquio Syndrome, has an incidence of 0,07 [64,88] to 3,62 [46] in 100.000 live births (reviewed in [47]). Unlike MPS III, which is almost exclusively a neurological syndrome, the skeleton is the main affected system in MPS IV, with the substrate accumulating predominantly in the cartilage and bones. Consequently, the major clinical manifestations observed are bone deformations, short stature, and mobility alterations [89]. In both cases, keratan sulfate (KS) and chondroitin-6-sulfate (C6S) are the accumulated substrates. So far, approximately 467 mutation have been described in the GALNS gene ([50]) **[50],** composed of 14 exons, all associated with MPS IVA [90,91]. Concerning type IVB, 263 ([50]) mutations are known to cause this disorder. The only FDA-approved treatment for MPS IV is elosulfase alfa (Vimizim®; BioMarin Pharmaceutical Inc.) that is used in MPS IVA patients. All other options are symptomatic and mostly consist in surgical approaches to prevent spinal cord damage or other skeleton issues, for example, spinal decompression surgery [92].

Yet another form of MPS, usually coined as Maroteaux-Lamy Syndrome, is MPS type VI (OMIM #253220). 242 mutations in the ARSB gene (which spans 8 exons) are known to cause this disorder ([50]). The estimated frequency for this disorder is 0,0132 [93] -7,85 [46] in 100.000 live births (reviewed in [47]). Even though being a multisystemic condition, MPS VI does not affect intelligence, and, like Morquio syndrome, the skeleton is the most affected system [94]. Thus, the clinical manifestations are very similar to those described above including short stature, low body weight and impaired pulmonary and motor functions [95]. To counteract the DS storage promoted by the deficiency of Arylsulfatase B (EC 3.1.6.12) activity, galsulfase (Naglazyme®, BioMarin Pharmaceutical Inc) is the drug approved and currently employed in patients. HSCT may also be possible; however, additional safety studies are needed [95–97].

MPS type VII (OMIM #253220) or Sly syndrome occurs with an estimated frequency of 0,02 [64,98–100] to 0,29 [55] per 100.000 live births (reviewed in [47]). Several systems/organs are involved in this disease with clinical features affecting organs as diverse as the eyes, lungs, heart, musculoskeletal, spleen, etc. Thus, the most common symptoms are described as coarse facial features, increased of cranial circumference, reduced of pulmonary function, obstructive airway disease, dystosis multiplex, decrease of mobility, joint contractures, abdominal abnormalities, short stature and hepatomegaly/splenomegaly. There may also be a neurological involvement as testified by recurrent observations of limited vocabulary and mental retardation in several MPS VII patients [101]. Overall, these symptoms are caused by an ubiquous accumulation of several different GAGs, namely DS, HS, and CS, as a consequence of the deficient activity deficiency of β-glucuronidase (GUS: β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31). The *GUSB* gene (12 exons) [102] with 81 mutations identified so far ([50]), is the one affected in this disorder [103]. The approved drug for this pathology is vestronidase alfa (Mepsevii™, Ultragenyx), which is indicated in both pediatric and adult cases [104].

Finally, MPS IX or Natowicz disease (OMIM #601492) is an ultra-rare disorder. The first report was published in 1996, with the described patient presenting a number of clinical manifestations associated to joint and skeletal systems [105]. This disorder is caused by a deficiency in the enzyme hyaluronidase 1 (HYAL1; EC 3.2.1.35) due to mutations in the *HYAL1* gene (3 identified until now [50]), which leads to the accumulation of yet another substrate: hyaluronan. Due to the rareness of the disorder, very few mutations have been reported to date (only 7), and a possible treatment is very challenging [106].

In general, even though the molecular bases and biochemical defects underlying MPS diseases are well defined, knowledge is still lacking on the pathophysiological mechanisms that actually trigger the appearance of different symptoms in the different organs and systems. And, even though much has been learnt over the last decades, from the study of

individual patients and, particularly, from the generation and extensive characterization of bona fide *in vivo* models, truth is we haven't still fully understood the whole physiological cascade, which underdies some of MPSs' most challenging phenotypes, namely those which affect the CNS. And this is particularly relevant since no therapeutic exists to ameliorate them. Still, finding an *in vitro* model that could recapitulate the disease-relevant features is also challenging once live neurons are inaccessible cells. Indeed, for almost a century, patient-derived fibroblasts were gold standard for *in vitro* studies in MPSs, as in all other LSDs. These cells were relatively easy to access, since a simple skin biopsy would be enough to obtain them and remarkably, they did display the hallmark subcellular/ intracellular phenotype that actually coined these diseases as "storage" disorders: the presence of undegraded or partially degraded substrates. Nevertheless, fibroblasts may also fail to recapitulate disease-relevant features, which are only expressed/evident in other particular cell types, of higher pathological significance such as neurons. A viable option is to generate the neurons from a patient-derived cell line, which involves extracting the cell from the patient and differentiating it into neuronal cells. Indeed, there are two possible ways to do this process: iPSCs and mesenchymal stem cells (MSCs) from the patient. 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364

## 4. Modeling Mucopolysaccharidoses with induced pluripotent stem cells (iPSCs) 365

Human iPSC generation in particular started its journey in 2007, when Yamanaka et al. [107] first generated those cells from human somatic fibroblasts using a remarkable method, which relies in the retroviral transduction of 4 independent transcription factors into patients' fibroblasts: Oct-3/4, Sox2, Klf4, and c-Myc. Remarkably, the cells that resulted from this experimental setup shared/showed numerous similarities with human embryonic stem cells (hESCs) including morphology, proliferation capacity, gene expression pattern and *in vitro* differentiation potential. Ever since this hallmark report was published, the search for novel and improved protocols for cells reprogramming advanced at an outstanding pace, with various optimizations being published in order to generate virtually every cell of interest from iPSC of different origins [108]. 366 367 368 369 370 371 372 373 374 375

Over the past few years, *in vitro* models derived from iPSCs have been unraveling some enigmatic aspects of MPSs. In particular, the subtypes that present neurological involvement appear as the ones with the greatest need for additional knowledge and new therapeutic solutions. 376 377 378 379

Here we will review numerous studies attempting not only MPS-derived iPSC generation, but also their subsequent differentiation into relevant cell types. We have divided those studies into four major groups, each one of them having a dedicated section in this review (**Erro! A origem da referência não foi encontrada.**). First, we will address the papers in which only iPSCs were generated, briefly discussing the methods used to characterize them. Then, we will focus on those papers where iPSCs were further differentiated into either neural precursor cells or totally differentiated neurons, highlighting the disease modeling potential of those lines by showing the numerous pathophysiological insights one can get with a few simple cellular assays. Then, we will go through the papers where those cells were used for *in vitro* drug screening, commenting not only on the results obtained but also on the advantages or disadvantages of the use of those particular cells for therapy development. Finally on the last iPSC-devoted section, we will refer to a few studies were the therapeutic potential of these particular SC was addressed. Meaning: we will summarize the papers where instead of generating iPSCs to further 380 381 382 383 384 385 386 387 388 389 390 391 392 393

understand one particular disorder or genotype or to serve as a drug screening platform, the authors have actually created them for gene therapy.

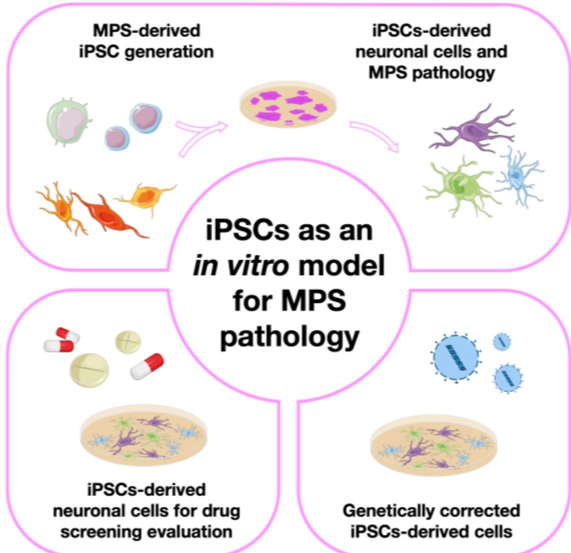


**Figure 1.** The four aims possible to achieve with MPS-derived iPSCs in vitro models. (Adapted from smart.servier.com)

*4.1. The basic studies: iPSC generation from different MPS patient-derived cell sources*

The first MPS-derived iPSCs were generated in 2011 when Thomas Lemonnier and colleagues [109] reprogrammed fibroblasts from two patients suffering from MPS IIIB into pluripotent stem cells (PSCs). As in any other iPSC generation report, the resulting SC were extensively analysed and characterized. In this particular study the authors confirmed a positive expression of three particular markers (SSEA4, Nanog and TRA-1-60) and the differentiation ability of those cells, thus proving their pluripotency nature. Additionally, the authors have also provided information on the karyotype presented by those cells. This is a relevant assessment whenever a novel iPSC line is generated but it should also be considered later on, when using the same iPSC line after several passages, or after having one particular iPSC cell line in culture for a long period. In fact, long-term iPSCs culture is known to result in chromosomal abnormalities, changes in gene expression and cellular functions, and even increases the risk of the iPSCs being tumorigenic. As genomic alterations present potential risks in the overall applications of iPSCs, it is crucial to monitor the genomic integrity of iPSCs lines. That is why iPSC karyotype analysis is such an important step on the validation of this type of cell models, and nowadays considered as a routine procedure by all the groups working with iPSC technology [109].

But these weren't the only published MPS IIIB-derived iPSCs reported in the literature so far. Two other MPS IIIB patient-derived iPSCs lines were generated from skin fibroblasts by Vallejo-Diez et al. in 2018 [110]. In that particular study additional pluripotency markers were also assessed besides the previously referred Nanog and TRA-1-60: Oct-3/4; Sox2; TRA-1-81 were also analyzed. As in the previous study, karyotype was also assessed, and the associated mutation confirmed. But the authors have actually went one step further in terms of SC characterization analysing the differentiation ability of the generated iPSCs by evaluating the formation of embryoid bodies after 10 days of differentiation, using specific markers from 3 germ layers. The same characterization was carried out for MPS IIIA-derived cells, where the same team was the first to create patient-derived iPSCs [111].

Regarding MPS IIIC, Noelia Benetò et al. have also generated iPSC lines, but this time using a slightly different protocol from the previously described ones. Instead of using patient-derived cell lines, these authors have created isogenic HGSNAT-mutated lines from healthy iPSCs using CRISPR/Cas9. This technology allows to create such lines in human cells which have the genetic background of the wild-type cells but differ by the genetic modification of interest. These isogenic pairs are powerful tools for understanding gene function. While circumventing confounding effects of genetic background, they allow for genotype-phenotype correlation studies [112]. To prove the reliability of this model, they measured HGSNAT enzyme activity and assessed the differentiation capacity of the generated SC. This last parameter, was studied by inducing the formation of embryoid bodies and their subsequent differentiation into three germ layers. The formation of these structures is a characteristic of pluripotent SC and serves as a platform for the intended differentiations [113].

Still, and even though the neurological involvement is a major hallmark of the sanfilippo syndrome, almost every other MPS may present with severe neurological symptoms/forms. Types I and II in particular have even specifically recognized clinical forms where the CNS is strongly affected, presenting with major clinical symptoms. Thus, these disorders would also strongly benefit from the development of appropriate neuronal cell models to study them. Furthermore, they would also allow for tissue- or cell-specific drug screening assays. Generating iPSC lines from those disorders is a rational step towards that first goal and that is probably one of the reasons why iPSCs lines from both disorders have also been created and subsequently published in the past few years.

Regarding MPS type II, in 2016, Eszter Varga et al. collected peripheral blood mononuclear cells (PBMCs) from phenotypically affected patients with 1-, 3-, and 7-year old [114–116] and an unaffected carrier mutation woman with 39-year old [117]. Then, all PBMCs patients' cells were subjected to induction of the pluripotent stage, originating disease-specific iPSCs, which were extensively characterized as expected/required by the technology itself.

Last but not least, MPS type I has also been modeled with the help of this revolutionary technology. In 2019, Lito S. et al. [118] and Suga M et al. [119] have reprogrammed and characterized dermal fibroblasts and PBMCs, respectively, into iPSC lines. The fibroblast-derived pluripotent cells were obtained from a patient with the Hurler form of the disease, whereas PBMCs were collected from a patient suffering from Scheie.

### *4.2. Moving one step further: generation of neuronal models from MPS-derived iPSCs*

As we have already referred, the neurological involvement, which places such a tremendous burden over patients suffering from several forms of MPS, may be further explored by differentiating iPSCs into different types of neuronal or pre-neuronal populations. And in fact, most works published up until now are not only focused on reprogramming different types of patient-derived cells (namely fibroblasts and PBMCs) but also on the differentiation step, searching for disease-relevant features in those cells. Ultimately, these models may also allow for the discovery of novel hallmarks related or non-related with neuropathology. Considering the intrinsic nature of all MPSs, lysosomal pathology is probably the more crucial parameter to study, once the enzymatic defect will primarily affect this organelle.

Thus, when it comes to disease phenotype assessments, some markers have been particularly relevant in the LSD field, namely the lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2). These two proteins are heavily investigated once it represents the major components of the lysosome membrane. For example, in the study

involving the first MPS-derived iPSCs [109], which were generated from MPS IIIB patients' samples, the accumulation of storage lesions was intensively analyzed through LAMP-1 and Golgi matrix protein 130 (GM130) detection. A prominent fluorescence of both markers was detected in patient-derived iPSCs, and the vesicles observed by microscopy were revealed to have a heterogenous content. This was actually the first study to describe Golgi Complex impairment in the MPS pathology. Most importantly, beyond iPSCs generation, this group has also investigated the differentiation into Neural Stem Cells (NSCs) by adding specific growth factors to the original iPSC culture, namely fibroblast growth factor 2 (FGF2), and endothelial growth factor (EGF). When this protocol was initiated, the development of neurospheres became evident and after 2 weeks of non-adherent growth, the authors measured both the expression of Nestin (a neural progenitor marker) and total GAGs storage. Interestingly, the higher LAMP-1 and GM130 expressions previously seen in iPSCs did not translate to the floating neurospheres. However, the gene expression profile showed significant alterations in several pathways including transducing extra-cellular, Wnt and transforming growth factor β (TGFβ) signals, as well as genes encoding proteins associated with cell adhesion, Golgi apparatus and lysosomes. Curiously, that higher LAMP-1 and GM130 fluorescence was seen again as soon as neurosphere adhesion was performed, and during the final process of neuronal differentiation. This observation was also accompanied by vesicle storage positive to LAMP-1 and Ganglioside GM3. These results reflect the existence of a modest cellular pathology during the neurodifferentiation of this iPSC model. This study was the first comprehensive characterization of MPS-affected neuronal cells *in vitro* [109].

To the best of our knowledge, the second report on the differentiation of MPS-derived iPSCs into neuronal cells, was the work of Bruyerè and collaborators, in 2015 [120], where these authors correlated two independent models of the disease: one *in vitro* and another *in vivo*. For the *in vitro* studies they used patient- and control-derived iPSCs, further differentiated into neural precursor cells (NPCs), while for the *in vivo* differences they used a mouse model. Their goal was to investigate the influence of HS saccharides accumulation in the focal adhesions (FAs). They saw that activation of FA occurred when neural cells from healthy individuals were submitted to exogenous soluble HS fragments. Consequently, this activation becomes constitutive in MPS IIIB, once those fragments are accumulated. Constitutive activation of FA, by its in turn, affects the polarization as well as the oriented migration of those cells [120].

Later, in 2015 Canals et al. [121] performed the differentiation of MPS IIIC-derived iPSCs into neuronal cells. Their goal was to verify if early functional alterations could be visible before the appearance of disease-related phenotypes. Briefly, iPSCs lines generated spherical neural masses (SNMs), whose expression patterns included PAX6, Nestin and Sox2. The existence of active neurons was also proven by the presence of microtubule-associated protein 2 (MAP2) and Synapsin (SYP), which are dendritic and synaptic markers, respectively. Besides the formation of mature neurons, an astrocytic-related marker Glial Fibrillary Acidic Protein (GFAP) was noticed. That observation further reinforced the neurogenic capacity of these cells. Regarding the neuronal cultures generated, as expected GAG accumulation was shown to have a progressive pattern, becoming significant only after 9 weeks. These observations document a marked difference between the patient's fibroblasts and iPSCs-derived neurons: the patients' fibroblasts presented a double amount of accumulated GAGs, right from the first cell culture, when compared to iPSCs-derived neurons. Networks activities were also evaluated to verify differences whether there were differences between Sanfilippo's- and the control- iPSCs-derived neurons. Through calcium imaging, the spontaneous activity of Sanfilippo-derived neurons was shown to gradually decrease between the 6 and 9 weeks. Concerning degradation of effective connectivity, which was determined by identifying causal influences among neurons through GTE, an information theory method

that allows drawing a functional map of neuronal interactions in the network, the authors reported that, quite differently from the controls analyzed, in the Sanfilippo neurons, strong connections were only established within a subset of neurons remaining the rest of them disconnected or poorly connected [121].

At a technical level, the authors used two different protocols, one relying on neuronal induction medium without any extra supplementation, and another where that medium was supplemented with N2 and B27, two chemically-defined supplements recommended for growth and survival of neuronal cells, and observed significant differences in the time it took for them to generate neurons. In fact, while it took several weeks in neuronal induction medium to arise mature neurons, when supplementing that same medium with N2 and B27, it took only 3-5 weeks to distinguish synapses between neurons. Moreover, the neuronal activity and effective connectivity analyses they performed were nicely designed and described, and could be applicable to virtually any other neurodegenerative disease in which iPSC-based models are available [121].

Five years later, Benetó et al. [122], took advantage of the existence of a few previously reported iPSC cell lines to generate neuronal and astrocytic models of Sanfilippo syndrome type C for disease modeling and drug development: two isogenic MPS IIIC mutant lines [95], one wild-type control (from a healthy donor), and one MPS IIIC-derived line [103]. Again, all four lines were differentiated into neurons and astrocytes through lentiviral transduction and promoting into the cells the overexpression of neurogenin 2 (Ngn2) in the case of neurons (named iNs) and Sox2/Nuclear Factor one B (NfIb) in the case of astrocytes (named iAs). To confirm cell identity, the authors performed a characterization of the specific markers: in the generated neurons, they detected an increase in neural stem cell markers, namely tubulin β-3 (TUBB3), SYP, MAP2, and Neuron-specific Class III β-Tubulin (Tuj1). In the astrocytes, they observed that the expression of astrocytic-specific genes namely GFAP, Aldehyde Dehydrogenase 1 Family Member L1 (ALDH1L1), calcium-binding protein B (S100B) and vimentin (VIM) increased during the astrocytic differentiation. In addition, disease-relevant features were assessed through LAMP-2 staining and HS quantification. On the LAMP-2 immunocytochemistry assays, the authors have clearly seen an intensity increase in all disease lines compared to the wild-type one. In the case of HS accumulation, they only present results for neurons, where, as expected, increased substrate storage could be observed [122] .

Still on Sanfilippo syndrome, for the most frequent type, MPS IIIA, a comprehensive study was carried out by R. J. Lehmann et al. in 2021 [123], to investigate the ability of fibroblasts-derived iPSCs to differentiate into a neuronal cell line and discover intrinsic mechanisms of the disease. After properly characterizing the pluripotency phase, the authors performed a neurodifferentiation protocol. Two main parameters were assessed: the FGF2 signaling pathway and the neurogenesis process. Interestingly, at the beginning of this study, a curious fact was noticed: when the FGF2 supplement was added to the medium, the proliferation rate of the MPS IIIA iPSC-derived NPC culture increased significantly. Remarkably, however, even with the supplementary-FGF2, the signaling pathway of this factor is still reduced, when compared to controls. So, understanding this event became a priority for this team. In fact, the FGF2 signaling pathway only occurs when this factor binds to a possible receptor. Since it also binds to HS, this may suggest that this GAG has a key role in neurogenesis and in the homeostasis of the CNS. Taking this into account, the subsequent step was to investigate the relationship between the accumulated HS in the MPS IIIA and that lower proliferation rate. They verified that the affinity of HS MPS IIIA to FGF2 was similar to the HS present in the positive control, meaning that the accumulation does not alter the affinity. So, a possible explanation for decreased FGF2 signaling is that once the FGF2 binds to the accumulated HS, it does not interact with the

proper receptors (cell-surface HS and Fibroblast growth factors receptors), thus affecting not only cell proliferation without supplementary FGF2 but also the signaling pathway. To investigate the disorder's impact on the neurogenesis process, control and disease cells were analyzed regarding both morphological parameters and expression patterns. At a structural level, the formation of cell bodies aggregation and cell extensions was seen in both cell lines. However, as already seen by other authors in SC models for other neurological disorders, in MPS IIIA cells, those characteristics were less frequent. Regarding the expression profiles, the genes evaluated were *Nestin*, *TUBB3*, Hyperphosphorilated neurofilament (*NF-H*), and neuron-specific enolase (*NSE*). In general, the increase/decrease pattern during the four weeks of neuronal induction was consistent between the controls and the disease cell lines; nonetheless, the disease cells showed consistently lower levels of all markers. This pattern was seen both in the absolute values themselves and in differences during the period of the procedure. Attention was also paid to the model's capacity to recapitulate disease-relevant features. So, the same parameters, which were initially assessed in fibroblasts, were also analysed after the neurodifferentiation protocol. Not surprisingly, the MPS IIIA cells exhibited higher levels of HS, a consequence of lower enzyme activity compared to the controls, further validating the disease modeling value of this kind of cells [123].

As previously stated, though, other MPS apart from the Sanfilippo syndrome may benefit from the development of disease-specific neuronal cell models, and from the pathophysiological insights one may gain from them. Thus, some of the most stricking reports on iPSC-derived neuronal and astrocytic models for MPSs, actually came from MPS II. In 2019, Kobolák et al. [124] have even proposed a novel neuropathology model using this approach. They used the iPSCs originally published in a number of publications already reviewed in the previous section [114–117] that were differentiated into NPCs and terminal differentiated neuronal cells. Briefly, those iPSCs were derived from two affected siblings. As expected, both individuals shared the same mutation, which results in an alteration of the open reading frame, which results in the appearance of a premature termination codon. Also included in this study was their mother, a carrier for the same causal mutation, and an unrelated patient with a different mutation (missense). Finally, the authors have also included cells from an unrelated non-carrier, which were used as a control. At the neurodifferentiation stage, neither the patients-derived nor the healthy cells had differences in the expression of specific neuronal markers. Briefly, for NPCs the authors assessed Nestin, Sox1, and PAX6; for terminal differentiated neuronal cells, on the other hand, they checked TUBB3, MAP2, and Neurofilament 200 KDa (NF200). An exhaustive characterization of those cells was done showing that mature neurons exhibited postsynaptic density protein 95 (PSD95) expression, an indicator of activated synapses. Astrocytes, on the other hand, were shown positive to GFAP and Aquaporin 4 (AQP4) markers[124]. In fact, the AQP4 channel is distinctly expressed in astrocytic membranes between the cerebrospinal fluid and brain parenchyma, and it is one of the major channels present in mammalian CNS [125]. Interestingly, according to these authors' results, the proliferation capacity of NPCs seems to be a distinctive factor between the controls and the patients' cells once, after 8 passages the proliferation capacity of the MPS II-derived cells slowed down or even stopped and the PAX6 and Sox1 expression decreased, independently of bFGF and EGF presence in the cell culture. Meanwhile, the control-derived NPCs maintained the proliferation rate up until passage 12. Actually, the authors considered this event to be related to the overall MPS II brain pathology: in normal conditions HS binds at a proper rate to transcription factors, not harming the proper function of these ones. However, in the case of storage, the accumulated HS usually binds at a higher rate to transcription factors, including the one with a key role in NPCs proliferation, FGF2. This overlink prevents the accomplishment of the transcription factor function. As a response, the cells start to differentiate into

neurons, occurring the appearance of anticipated neurites when compared with control cells [124].

One of the essential aims of this work was to verify if some of the disease hallmarks were already present in the NPC stage. Thus, the authors have performed several analyses and, remarkably, they realized that GAG accumulation was not evident. Interestingly however, it was even reduced compared with both controls (carrier and non-carrier). They hypothesized that this phenomenon could be related to the lower levels of the early endosomal marker RAB5, (which is translated in a lower endocytosis level) and to the normal levels of the late endosomal marker RAB7, and of the lysosomal marker Cathepsin D, in addition to the higher LAMP-2 expression. The existence of those factors is reflected into functional exocytosis by patients' cells: GAGs and GAG fragments are expelled to the extracellular space, which could explain the appearance of GAG accumulation in cerebrospinal fluid. This whole pattern changed however, when mature neurons and astrocytes were analyzed. In fact, for those mature neurons and astrocytes differentiated from cells harboring the frameshift/PTC mutation, GAGs accumulation was (quite) evident. Importantly, however, the levels of Rab7, Rab5, and LAMP-2, were still similar to those observed in controls, indicating a non-influence of endosomal-lysosomal system over substrate accumulation. It should also be stressed that for the cells harboring the missense mutation, all assessed parameters were comparable to those seen in the controls [106]. While somehow unexpected, these results highlight the intrinsic potential of these sort of cell-based patient-derived models as they allow for more accurate comparisons between the effect of different disease-causing mutations over several subcellular parameters, ultimately allowing for more precise genotype-phenotype correlation. Also noteworthy, regardless of the analyzed genotype, all terminal differentiated neuronal cells (neurons and astrocytes) showed a significantly increased of the autophagy marker LC3-I, revealing alterations/the involvement of this pathway in disease cytopathology. Additionally, an accumulation of autophagosomes, as well as a lower ratio of LC3-II/LC3-I, was also detected [124].

Regardless of the cell differentiation status, a common point in the cytopathology of MPS II from NPCs and TDs was the presence of ER stress with the occurrence of dilated ER cisterns. In NPCs, the authors have observed a significantly higher level of XBP1, a well-known ER stress marker. For TDs, even more events related to this stress were observed, namely: depletion of ER luminal Ca2+ storage, higher ion concentration in the cytoplasm, and a higher sensitivity to apoptosis. Concerning cell death, they noticed a higher rate of apoptosis in astrocytes rather than other TDs. It is known that this cell type plays an important role in supporting the differentiation and survival of cortical neurons. Therefore, if they are not functional, cell death and neurodegeneration may occur [124].

Also in MPS I, a few studies exist where iPSCs were differentiated into NSCs and from where curious insights were gathered. An interesting study was performed in 2018, by Swaroop et al. [126], where after generating iPSCs and NSCs from all MPS I subtypes, the authors addressed the question of whether those three subtypes could be distinguished from each other, while extensively characterizing each one of them. In the characterization step, they observed a normal iPSCs and NSCs morphology, karyotype, and growth rate in all three. Still, differences among the MPS I subtypes were quite evident, when it came to the disease's hallmarks. Regarding enzyme activity, for example, all NSCs-MPS I types exhibited a lower rate when compared with controls. However, the levels observed in the Hurler-derived cells were remarkably lower than the others. The same happened when DS and HS accumulation and lysosomal enlargement were evaluated: the values for the Hurler subtype were much higher. Also noteworthy, when the authors compared those cell lines by differential expression (DE), about 3036 genes were found to be significantly changed between patients and controls. Remarkably, however, out of those, 42% were

Hurler Syndrome exclusive. Not surprisingly, those genes were involved in GAG homeostasis, dysregulation of the lysosomal pathway and autophagy [108]. Overall, these results strongly supported the idea that one can nicely characterize and distinguish different forms of the same disorder, by evaluating iPSC-derived models, as they recapitulate at the subcellular level the severity we see in patients [126].

Four years later, another interesting study was conducted by Lito S. et al. [127] focusing on the most severe form of the disease alone (Hurler). In that paper, besides reprogramming dermal fibroblasts into iPSCs and generating NSCs, the authors went one step beyond and created an isogenic control from these iPSCs by reestablishing IDUA expression to avoid any type of variability that could emerge from the comparison with iPSC control cell lines derived from other individuals. Then those isogenic cells were also differentiated into NSCs. As a matter of fact, these cells showed a total functional enzyme both in iPSCs phase as well as when differentiated into NSCs. Through comparison with isogenic ones, they could see the most evident marker of these disorders: GAG accumulation. Furthermore, at the end of a three weeks-neuronal differentiation protocol (where FGF2 and EGF were removed from the media), they saw a higher migration *in vitro* of rescued-enzyme NSCs as well as neurite outgrowth when compared to deficient iPSCs-derived NSCs. In turn, proliferation capacity during three weeks of neurodifferentiation, did not change significantly between the two cell conditions. They hypothesize that due to the strong binding properties of CS and HS when accumulation occurs, these storage products bind to molecules responsible for neurite outgrowth and cell migration, preventing their binding with the proper receptors, and accomplishing the right function. Also, these aspects were accompanied by an evaluation of gene expression patterns. Biological processes associated with pathways of TGFβ, focal adhesions, PI3K-AKT signaling, Hippo signaling, RAP1, extracellular matrix interaction, and calcium signaling were altered with around 173 downregulated and 167 upregulated genes. In general, these migration defects and gene expression changes seen in patients affected by monogenic diseases are associated with a cause-effect relationship, where the genotype presents as a cause and the phenotype as an effect. However, based on these results, the authors purpose that the reverse may also occur, presenting a bidirectional pattern [127].

### *4.3. iPSCs-derived neuronal cells for drug screening/ therapies evaluation*

As we have already referred, MPS iPSC-derived neuronal cells have been generated not only to model MPS and study their pathology. Indeed, one of their crucial goals is to work as a platform to test future therapeutics. Thus, several research and development groups, some of them already mentioned in the previous sections, have been using those cells to test a number of compounds that may allegedly hold promise for the treatment of this LSD class.

Starting, again, with the Sanfilippo syndrome, one of the studies referred before [122], besides intending at the development of neuronal and astrocytic models derived from MPS IIIC iPSCs, also aimed at testing an SRT approach that had already given positive results in MPS IIIC fibroblasts [128]. That was the work of Benetó and co-workers, back in 2015, and the approach they wanted to test consisted on the use of a siRNA against one of the genes responsible for GAGs biosynthesis (the *EXTL2* gene) as a genetically triggered SRT. Still, while its application in the generated neuronal and astrocyte cells revealed a great success in the reduction of mRNA levels of this gene (about 75%), when the HS levels were analyzed in neurons, no difference in substrate accumulation could be detected. Curiously, this parameter was not measured in astrocytes, and it is actually a future perspective of this the group to test it as well. A few years ago, this team has also reported an siRNA-driven SRT approach against *EXTL3* (another gene involved in GAGs biosynthesis) with positive results in fibroblast disease cells [128], and they proposed to

assess its effect in the same neuronal and astrocytic models but, to the best of our knowledge, no follow-up studies have been published so far. Altogether, however, the results they published so far, further highlight the need to develop suitable cell models for drug testing, by clearly demonstrating there may be significant differences between the results obtained *in vitro* in fibroblasts vs neurons using the exact same therapeutic molecule. In fact, fibroblasts are the classical human cellular model in LSDs, but there are significant metabolic differences between fibroblasts and neural cell types. Furthermore, fibroblasts are dividing cells, while neurons are not. This means that even though fibroblasts accumulate undegraded materials, storage can be underestimated due to dilution by cell division, when compared with that of non-dividing cells [122]. 740 741 742 743 744 745 746 747 748 749

One year later, Huang W. et al. [129] published a comprehensive work, which went all the way from the iPSCs generation and characterization up until the generation of (iPSC-derived) MPS IIIB neuronal cells. While it goes far beyond the scope of this review to go through the extensive characterization analysis and pathophysiological assessments the authors performed on both types of cells, we would like to briefly highlight the therapeutic assessment they made *in vitro* using these models. Briefly, they examined the effects of three possible therapeutic agents: ERT with recombinant NAGLU (rhNAGLU), δ-tocopherol (DT), and hydroxypropyl-β-cyclodextrin (HPBCD). When rhNAGLU was applied to NSCs, a dose-dependent decrease in enlarged lysosomes was readily observed; the same happened when testing DT and HPBCD in addition to a dose-dependent reduction in the lipidic accumulation. In fact, those two compounds have already positive results in Niemann-Pick disease type C and, more recently, also in other LSDs [130–132]. Due to this observation, both compounds were also evaluated in MPS II iPSCs-derived NSCs by Hong et al. [133]. In the case of DT, the results showed a reduction of lipid accumulation after three days, but in a dose-dependent manner; in turn, when evaluating the lysosomal accumulation it was revealed only a 7% reduction. The HPBCD results were not so encouraging, once it had virtually no effect on primary and secondary accumulation. As previously anticipated, however, when NSCs were treated with recombinant enzyme for MPS II (rhIDS), a marked reduction of lipid accumulation was also observed. 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769

Curiously, the effect of rhIDS enzyme was also the target of a study developed in 2018 by Rybová et al. [134]. This study also contemplated reprogramming MPS II PBMCs into iPSCs and their subsequent differentiation into NPCs, neurons, astrocytes, and oligodendrocytes. Having all those cells properly characterized, the authors moved on to evaluate the effect of rhIDS over GAG levels. Remarkably, however, their results showed that despite achieving 10-fold higher enzyme activity levels, the treatment could not reverse the exponential growth of GAGs levels, even though some decrease could be seen [134]. 770 771 772 773 774 775 776 777

### *4.4. Genetically corrected MPSs-derived iPSCs* 778

Finally, we will also mention a few studies, which have provided *in vitro* proof of principle on the potential of *ex vivo* genetically-corrected iPSCs for therapeutic purposes. 779 780

The proof of concept study on the therapeutic use of iPSC for autologous HSCT was published in 2015, by Griffin and co-workers, who attempted *ex vivo* gene therapy using patient iPSC-derived NSCs to reverse brain pathology in MPS VII [135]. Those authors assessed the engraftment potential of MPS VII NSCs genetically corrected with a transposon vector, by transplanting those cells in a previously reported mouse model for the disease, the so-called NOD/SCID/MPS VII model. Briefly, they injected intraventricularly genetically corrected GFP-labelled NSCs into different neonatal mice populations, either suffering or not from MPS VII. Remarkably, the authors observed 781 782 783 784 785 786 787 788

similar levels of cell distribution in both pathological and non-pathological contexts, demonstrating that engraftment properties are not influenced by disease. Importantly, transplanted cells survived and remained in the immature stage (Nestin-positive) for over 4 months. However, the proliferation rate reduced dramatically with the total disappearance of proliferation markers after 4 weeks of transplantation. It is worth mentioning that the authors chose to work with neonatal mice for these initial assessments because they provide a more hospitable environment for engraftment relative to the adult brain. Then, to test whether similar results could be obtained in older animals, they injected *ex vivo* corrected MPS VII iPSC-NSCs in diseased mice adult brains. Again, the immature stage remained with a Nestin-positive pattern. In the adult mice, however, the authors also addressed a number of pathology aspects, in order to address the therapeutic potential of this approach. And, in fact, they did detect GUSB activity but only near to the injection site of the hemisphere receiving corrected cells. Additionally, they also verified a high reduction in neuroinflammation after only 1 month of transplantation in that same region. Basically, they showed that xenotransplantation of *ex vivo* corrected MPS VII-derived NSCs into a mouse homolog of the human disease, can reverse pathologic lesions surrounding the engrafted cells. But, more relevant than the particular results they saw in this disease and their accurate analysis, is the innovation potential they hold and the new avenues they open, by showing that genetically corrected iPSC-derived NSCs may indeed may have potential to treat MPSs [135].

Then, in 2018 Clarke et al. described a somehow similar approach, attempting to use genetically corrected NSCs derived from iPSCs as a transplantation approach to the treatment of MPS IIIB [136]. Briefly, *Naglu*$^{-/-}$ mouse embryonic fibroblasts were reprogrammed into iPSCs and later differentiated in NSCs. Those cells were then corrected *ex vivo*, through lentiviral transduction of the full-length human *NAGLU* cDNA. This lead to an obvious overexpression of the gene in the corrected NSCs, which resulted in a 4-fold increase in enzyme activity and in a 14-fold higher level of secreted NAGLU when compared with wild-type. Importantly, before they attempted HSCT of those genetically corrected cells, the authors confirmed *in vitro* whether secreted NAGLU could enter in Naglu$^{-/-}$ cells in an M6P-dependent way, and verified that corrected cells were indeed able to "cross-correct" enzyme-deficient ones. Additionally, they also addressed whether there was a difference in lysosomal enlargement between genetically corrected NSCs and unmodified Naglu$^{-/-}$-derived NSCs. Curiously, they could not see any differences. However, when both cell lines were allowed to differentiate into mature neural cells, the ones derived from genetically corrected NSCs did show a significant decrease. Only then did the authors move to *in vivo* studies. Basically, they did virtually the same previous teams had done before: *ex vivo* genetically modified cells were injected into newborn *Naglu*$^{-/-}$ mice to understand whether they would promote an amelioration of the animals' phenotype. But there is one remarkable aspect about this study that shoud be highlighted: this team has evaluated two independent protocols: intracerebroventricular (ICV) and intraparenchymal (directly in the striatum), and the pathological aspects they analysed were microglial activation, astrocytosis, and lysosomal dysfunction/storage material. All these aspects were analyzed through immunostaining of CD68, GFAP, and LAMP-1, respectively [136].

Again, we will not review in detail all their observations, but we would like to stress that, from this team's observations regarding the two administration routes attempted, intraparenchymal was the one shown to have better engraftment. Still, it should be stressed that, at 2 months of age, there was high variability in the pathophysiology results in both ICV and intraparenchymal approaches. Importantly, however, the follow-up results after long-term transplantation of the corrected NSCs into *Naglu*$^{-/-}$ mice were much more evident. The evaluation of the long-term effect was performed after 9 months of transplantation with the intraparenchymal administration route. In general, NAGLU

activity was detected in the majority of engrafted animals. Furthermore, all pathological hallmarks evaluated were more pronounced in non-transplanted *Naglu*$^{-/-}$ mice. In grafted *Naglu*$^{-/-}$ mice, however, CD68, and GFAP levels were significantly lower in some regions of the brain. A similar pattern was observed after LAMP-1 staining, meaning that transplanted mice showed a significant decrease in storage material, a reduction in astrocyte activation, and complete prevention of microglial activation within the area of engrafted cells and neighboring regions, with beneficial effects extending partway along the rostrocaudal axis of the brain. Altogether, this study provided evidence that the transplantation of genetically corrected iPSCs-derived NSCs, may indeed represent a popential treatment for MPS IIIB and this is particularly relevant since no approved therapeutic approach exists for this neurological MPS [136][137].

The latest *ex vivo* gene therapy experience to be performed in MPS models is extremely recent. It was pubished in 2022 [137] and took advantage of results we have just reviewed, for MPS IIIB [136]. In fact, the same team, which originally published the proof of principle on the potential of *ex vivo* corrected NSCs to positively impact the brain neuropathology in *Naglu*$^{-/-}$ mice, later extended that study by using a modified Naglu enzyme with the fusion protein IFGII (named NAGLU-IGFII) for the *ex vivo* correction of the NSCs. This modified/chimeric enzyme, had already been described to allow a greater cellular uptake via IGFII binding sites on the mannose-6-phosphate receptor (M6PR). Again, the overall process of NSCs generation was performed as well as their lentivirus transduction of the NAGLU-IGFII sequence. Having confirmed that the modified NAGLU-IGFII enzyme could also be secreted and taken up, just like the unmodified enzyme they had previously reported [136], the authors moved on to *in vivo* studies. Briefly, they engrafted modified cells into the brain of newborn mice and evaluated the long-term therapeutic effect of that approach, 9 months post-transplantation. First, they confirmed the remaining capability of engrafted NSCs to generate different subtypes of CNS-associated cells through positive staining of several markers: NeuN and MAP2 for neurons; GFAP for astrocytes; and O4 for oligodendrocytes. Once more, the success of the engraftment could be better since there was a high variability in the enzyme activity between sections of the brain in different animals. However, the range of enzyme activity was increased by 10%, compared to *Naglu*$^{-/-}$ mice, which could be promising once it is reported that sometimes only an increase of 1-5% is sufficient for a proper enzyme activity correction. In the case of pathophysiological events, glial activation and storage accumulation, measured, respectively through the staining of CD68/GFAP and LAMP-1, revealed a pattern similar to that of wild type animals. Both effects were more pronounced in closer injection sites [137].

Furthermore, the authors also assessed a parameter, which had not yet been looked at in previous studies: the downregulation of MAP2. MAP2 is now known to have a relevant/significant role in the microtubule stabilization of dendritic processes. Its downregulation is heavily associated with dementia in Alzheimer's disease. Dementia is also a primary symptom in MPS IIIB and, remarkably, when *Naglu*$^{-/-}$ mice were stained for MAP2, the results have shown that MAP2 was reduced when compared with wild type. 9 months post-transplantation, this downregulation was actually reversed, with treated animals presenting MAP2 levels similar to those observed in *Naglu*$^{+/-}$ mice. Moreover, the accumulation of aggregates of synaptophysin, which is a known indicator of axonal damage in inflammatory conditions, was higher in *Naglu*$^{-/-}$ mice than in wild type and engrafted animals [137].

Overall, even though the efficacy of this therapeutic approach must be improved to reach all brain sections and counteract the Sanfilippo-associated neuroimmune response throughout the whole brain, truth is that, once more, this team has gathered evidence on the possibility of *ex vivo* gene therapy, with remarkable ameliorated MPS IIIB phenotypic

aspects. Moreover, this was the first report documenting a significant reduction ofthe neuronal marker Map2 and accumulation of synaptophysin-positive aggregates, both well-known to be related with neuropathophysiology [137].

Then again, even MPSs, which already benefit from the existent ERTs, may ultimately benefit from this sort of approaches. Therefore, *ex vivo* gene therapy experiments have also been performed in MPS I. In fact, in 2019, Miki et al. [138] have generated iPSCs from *Idua*$^{-/-}$ mouse embryonic fibroblasts. Then, the authors performed the *ex vivo* correction of those cells by CRISPR/Cas9 technology and verified that the resulting levels of enzyme activity were significantly restored with values comparable to the wild-type iPSCs. While exploratory and not yet attempted *in vivo* these results further validate the overall potential of iPSCs and iPSC-derived cells for gene therapy in MPSs.

Table 2 summarizes the works performed until the moment with iPSCs technology.

**Table 2.** Works performed in MPSCs using iPSCs technology

| Disorder | Affected gene | Defective Enzyme | Stored substrate | Subtype | Generation of MPS-derived iPSCs | | | | Drug Screening | Ex vivo gene therapy |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Source | iPSC | NPC | Mature Neurons | | |
| MPS I | *IDUA* | α-L-iduronidase | DS and HS | Hurler | Fibroblasts | [118,126,127] | [126,127] | | | |
| | | | | | Mouse Embryonic Fibroblasts | [138] | | | | [138] |
| | | | | Hurler/Scheie | Fibroblasts | [126] | [126] | | | |
| | | | | Scheie | Fibroblasts | [119] | | | | |
| | | | | | PBMCs | [126] | [126] | | | |
| MPS II | *IDS* | Iduronate-2-sulfatase | DS and HS | | Fibroblasts | [123,133] | [123,133] | | [133] | |
| | | | | | PBMCs | [114–116,134] | [124,134] | [124,134] | [134] | |
| MPS III | *SGSH* | Sulfamidase | HS | A | Fibroblasts | [111] | | | | |
| | *NAGLU* | α-N-acetyl-glucosaminidase | | B | Fibroblasts | [109,110,120,129] | [109,120,129] | [129] | [129] | |
| | | | | | Mouse Embryonic Fibroblasts | [136,137] | [136][137] | | | [136,137] |
| | *HGSNAT* | N-acetyltransferase | | C | Fibroblasts | [113,121] | [121,122] | [121,122] | [121,122] | |
| MPS VII | *GUSB* | β-Glucuronidase | DS, HS, and CS | | Mouse Embryonic Fibroblasts | [135] | [135] | | | [135] |

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## 5. An alternative approach to model Mucopolysaccharidoses 912

Regardless of its ultimate purpose, in general, the rationale followed in all the studies reviewed so far is the same: first, differentiated cells from patients with the target disease are reprogrammed into iPSCs and, then, differentiated again but into disease-relevant cell lines, thus creating a viable cell model for neuronopathic MPS. This technology, as described above, is undoubtedly contributing to increase the knowledge on the pathophysiology of MPSs with neurological involvement and, consequently, with no treatment available. Nevertheless, while iPSC technology proves to be quite valuable and promising, it also involves some disadvantages. Those positive and negative considerations are recapitulated in the Figure 2. 913 914 915 916 917 918 919 920 921

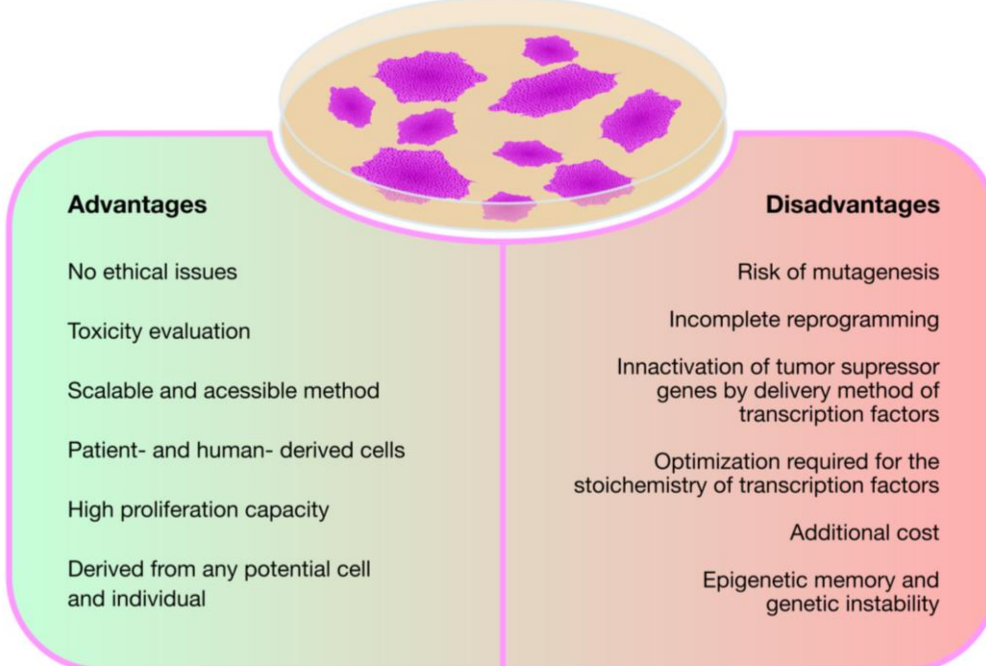


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**Figure 2.** Advantages and limitations of iPSCs. 923

That is why, alternative protocols and additional sources of SC should also be considered, especially those, which are naturally-occurring (Figure 3). 924 925

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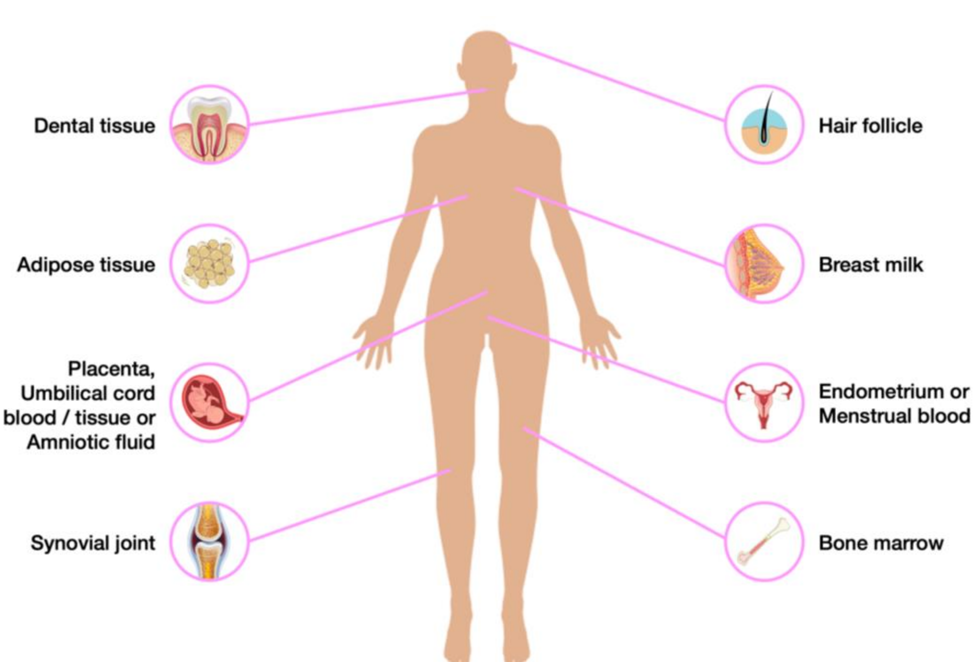


**Figure 3.** Different sources of Mesenchymal Stem Cells (MSCs) adapted from Liu et al., 2022 [139]; Fridman et al., 2018 [140], Macrin et al., 2017 [141] (adapted from biorender.com). 931 932

An excellent option would be to take advantage of patients' MSCs, reducing the possibility of errors and avoiding the long, laborious and expensive pluripotency induction phase. In fact, those cells represent a suitable alternative once they can be differentiated into any of the three germ layers: endodermal, mesodermal, and ectodermal, as long as they are cultured in proper media. To be considered a MSC, the cell needs to fulfill a number of criteria (Figure 4). 933 934 935 936 937 938

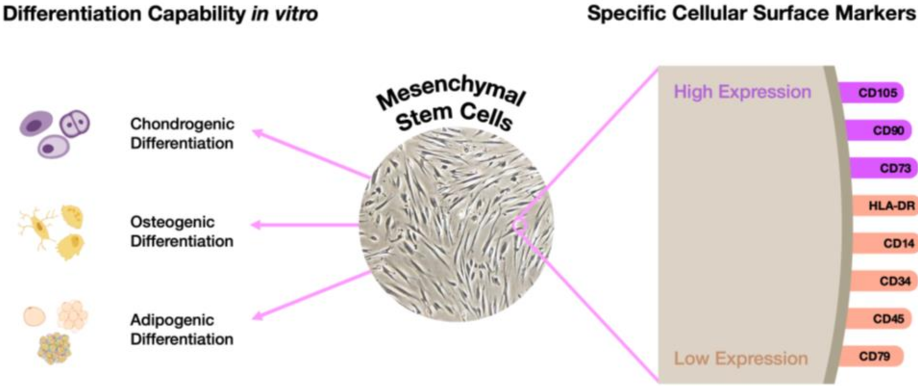


**Figure 4.** Minimal Requirements for identification of MSCs (adapted from biorender.com) 942

Bone Marrow Mesenchymal Stem Cells (BMMSCs) are the more often used ones. However, the patient's wellness remains an essential issue, due to invasive procedure [140,142]. 943 944 945

An interesting study [143] in 2000 introduced to the world a possible new source of SC: the dental pulp. The dental pulp is an oral non-mineralized tissue with various cell types, 946 947

localized in the central pulp cavity and mostly comprises soft tissue with nervous/vascular lymphatic elements [144]. Inside it, we may find the so-called Dental Pulp Stem Cells (DPSC). Those cells have an ectodermal origin derived from neural crest cells [145], more specifically from peripheral nerve-associated glia [146].

In that original study [143], those recently discovered SC were compared to BMMSCs, and the evidence they gathered showed that those DPSCs exhibit a higher proliferation rate when compared to BMMSCs, while expressing the same pluripotency markers. Thus, this pivotal study became a launching pad for the subsequent exploration of these cells. The impossibility of generating adipocyte cells in the original study was the only lack in classifying DPSCs as MSCs. However, over the following years, more evidence was gathered proving their stem nature. Ultimately, in 2002, the same group that originally assessed their MSCs features, was actually able to promote the adipogenic differentiation of those cells using a more specific induction medium. They also confirmed that human DPSC are capable of self- renewal after an *in vivo* transplant [147].

After a few years of constant research, a terminology was established that is still used today, which allows us to distinguish between the different SC populations that reside inside the dental pulp (Figure__). Indeed, depending on the source of the oral cavity from which they are extracted, five different types of stem cells may be distinguished: DPSCs, Stem Cells From Deciduous Teeth (SHEDs) [148], Stem Cells From Apical Papilla (SCAPs) [149], Periodontal Ligament Stem Cells (PDLSCs) [149], and Dental Follicle Stem Cells (DFSCs- precursor cells of PDLSCs) [149–151].

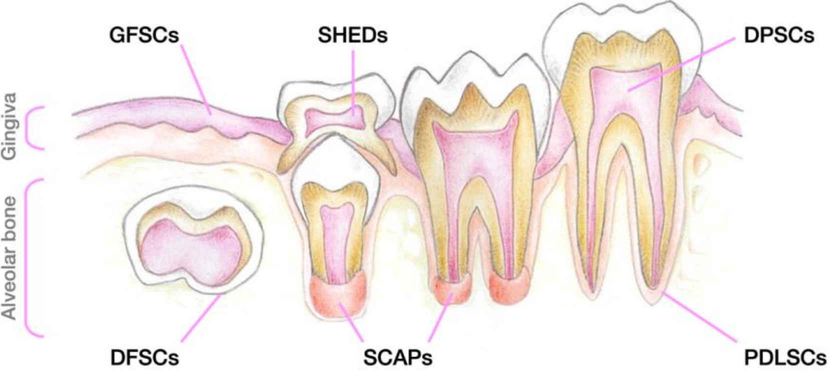


**Figure 5.** Principle sources of Dental Mesenchymal Stem Cells in oral cavity.

Besides the different oral cavity source, we can distinguish those SCs by their proliferation rate and potential to differentiation into the several cells. Regarding the proliferation rate, the Follicle-derived ones seem to have the highest, closely followed by SHEDs, SCAPs, PSLSCs and DPSCs [152–159]. In Table 1_ , we review some experiences done so far, to identify the better cell type for each kind of differentiation.

**Table 1.** Potential Differentiation of the different Stem Cells derived from the Oral cavity

| Type of Differentiation | Potential Differentiation | References |
|---|---|---|
| Osteogenic | PDLSCs>DFSCs/SHEDs>DPSCs>SCAPs | [148,153,159–162] |
| Chondrogenic | DPSCs>SCAPs/DFSCs/PDLSCs | [153,161,162] |
| Adipogenic | DFSCs>DPSCs/SCAPs>PDLSCs | [153,162] |
| Neurogenic | SHEDs>PDLSCs>DPSCs>DFSCs>SCAPs | [161,163,164] |

We are now in 2023, and the existence of these different SCs populations has now been known for over 20 years. So, DPSC and SHEDs in particular, have been generated from pulp for some time. Nevertheless, the majority of the studies involving those cells have focused on their differentiation into chondrocytes for dental repair, with the eventual goal of re-growing teeth from multipotent dental mesenchymal stem cells (DMSC) cultures [147][165]. Also addressed by a few teams is the potential they hold for stroke therapy. The first study to investigate DPSC in an animal model of stroke dates back to 2009 and used a mechanical extraction method to obtain cells from human third molars. The cells extracted from those teeth were shown to efficiently express the nuclear receptor related 1 protein, which is essential for the dopaminergic system of the brain, and promote, when transplanted, motor functional recovery [166]. After this pivotal study was performed, a few others followed, always relying on the use of SC from different dental pulp sources, and being tested *in vivo* in rat models of focal cerebral ischaemia via the transient occlusion of the middle cerebral artery. While it falls completely out of the scope of this review to summarize all those studies, it is worth mentioning that most of them showed really promising results (reviewed in [167]). Curiously, those cells have been shown to enhance poststroke functional recovery through a non-neural replacement mechanism, i.e., via DPSC-dependent paracrine effects ([168]; reviewed in [167]). And that is probably one of the reasons why this sort of cells have been addressed for their therapeutic potential on many other disorders, affecting various different organs such as kidney (acute renal injury [169,170] and nefritis [171]); lungs (acute lung injury [172]); brain (Parkinson's disease [173,174],Alzheimer's disease [175], cerebral ischemia [176,177]); spinal cord (spinal cord injury [178–181]); liver (liver fibrosis [182–184]); heart (acute myocardial infarction [185,186]); muscle (muscular dystrophy [187–189]); bone (calvarial defect [171,190–192], and osteoporosis [193]); skin (wound injury [194,195]); pancreas (diabetes [196,197]) eye (glaucoma [198], cornea trauma [184]) and immune system (rheumatoid arthritis [199], autoimmune encephalomyelitis [200] and systemic lupus erythematosus (reviewed in [201,202]).

And if it is true that, for most of these injuries, the evidence gathered so far comes from *in vivo* studies alone, when it comes to the use of DMSC in oral diseases, the scenario is significantly different, with 2 clinical studies on pulp regeneration having been launched within the past several years that have achieved breakthroughs in humans (reviewed in [201]). Overall, the results are so good and the possibilities so vast that soon a commercial interest was found in this type of cells. In fact, due to their easy accessibility and favorable therapeutic applications, cell/tissue banking in the dental field are now a reality in several countries, with some of the most well-known ones being BioEDEN (Austin, Texas), Store-a-Tooth (Lexington, Kentucky), Cell Technology (Japan) or the Tooth Bank (Brownsburg, Indiana) (reviewed in [158,203]). And as exciting as these results and perspectives may

sound per se, we believe that the overall potential of these SCs goes far beyond their properties for tissue repair and regeneration. We think, as other authors have also highlighted before, these cells also hold an exceptional potential for neurogenetic disease cell modeling and basic research. In general, DMSC have a neural crest origin, which makes them a useful source of primary cells for modeling virtually any neurological disorders at the molecular level [204]. Given our interest in LSDs, their monogenic nature and the extremely high prevalence of severe neurological phenotypes in this group of disorders, we considered DMSCs as a perfect model to study these disorders.

Interestingly, while their modelling potential has never been addressed for LSDs, as advantageous as it may sound, truth is DPSC are not totally unknown in the field. In fact, back in 2015, Jackson et al. [205] suggested that human MSCs derived from Bone Marrow and Dental Pulp could work as an alternative to the use of Hematopoietic Stem Cells, in standard transplantation approaches for the treatment of MPSs. Similarly to what has been discussed in the last session in which we summarized the studies published so far in MPS using iPSCs, in this particular publication, it was the therapeutic potential of the MSC per se, which was analysed. Actually, none of the MSCs analyzed derived from MPS patients. Instead, all studies were performed in MSCs obtained from healthy donors. This meant that neither the BMMSCs nor the DPSCs they established had any MPS-related enzymatic defect. Instead, all analysed cell lines (MSCs and HSCs) were able to produce the different MPS-associated enzymes in the cell layer and secrete low levels of each and every one of them into the surrounding media, the same being true for the used HSCs. However, MSCs were found to produce significantly higher levels of the majority of MPS enzymes assayed when compared to HSCs, a result that can be considered particularly relevant for therapeutic purposes.

But these authors have done more than just characterizing the normal levels of MPS-related enzymes secreted by different types of wild type SCs, namely BMMSCs, DPSCs and HSCs. They also attempted to overexpress, through lentivirus transduction, four different lysosomal enzymes in those same cell lines, to check whether their secretion levels were somewhat similar. Importantly, the evidence they gathered further supported the idea that MSCs had higher secretion and production levels of MPS enzymes when compared to HSCs. Also noteworthy, the lentivirus transduction was more efficient in MSCs compared with HSCs.

Then, the authors moved on to investigate *in vitro* the cross correction potential of MPS enzymes secreted from those two different sorts of MSCs in MPS patients' derived fibroblasts, and after confirming the reduction of GAGs accumulation, they also verified that this cross-correction was reached in an M6P-dependent way.

Finally, they also addressed the differentiation ability of the MSCs tested, verifying that both transduced and non-transduced cells maintained that capacity, with only slight differences in the neurogenic process, which appeared to have a slower differentiation pattern in transduced MSCs. As expected, however, MSCs derived from dental pulp had a premature upregulation on mature neuron markers, when compared with those derived from bone marrow.

Altogether, these results provided the *in vitro* proof of principle on the therapeutic potential of DPSCs and BMSCs as an isolated therapy or even combined therapy with the standard HSCTs. To the best of our knowledge, no follow-up studies or *in vivo* assessments have yet been published on this subject, even though its overall results seem extremely promising.

Besides the different oral cavity source, we can distinguish those SCs by their proliferation rate and potential to differentiation into the several cells. Regarding the proliferation rate, the Follicle-derived ones seem to have the highest, closely followed by SHEDs, SCAPs, PSLSCs and DPSCs [152–159]. In Table 1_ , we review some experiences done so far, to identify the better cell type for each kind of differentiation. 1070 1071 1072 1073 1074

To the best of our knowledge, MPS patient-derived DPSC had never been used for differentiation into specific cell types even though they represent a natural source of SC that may be used to investigate human disease especially for the infantile forms of these disorders. In fact, taking into account that the most severe forms of MPSs are pediatric, there is one particular population of SC in the dental pulp that seems particularly suitable to study them: SHEDs. Among their numerous advantages, which include a high proliferation rate and the greater tendency to generate both skeletal and brain cells, SHEDs collection does not require the active removal of teeth, only their natural fall, and this is certainly an advantage for children who may already be dealing with undue stress and pain. 1075 1076 1077 1078 1079 1080 1081 1082 1083 1084

It is also worth mentioning that, while this review focuses on the insights one can get over the neuropathology of MPS by studying iPSCs, and we have only commented on the neuronal differentiation potential of SC from different sources, such as the DPSC, their differentiation capacity to osteogenic fates is also known and successful protocols are published. This is quite relevant for MPS disease modelling because some of these diseases present with a marked skeletal phenotype, which fails to be corrected by the currently available therapies. Thus, by implementing the method here envisaged, one may also pave the way for additional applications of DPSC. For example, we may easily foresee their differentiation into chondrocytes, one of the major components of cartilage and primary site of accumulation in several LSD. 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094

In general, the higher the number of genotypes we collect the larger the spectrum of future applications our DPSC-derived LSD neuronal cultures may have not only in our lab but also for other researchers in the field. In addition, with the advances of new gene editing technologies, such as CRISPR/Cas, base editing, prime editing and the "older" transcription activator-like effector nucleases (TALEN) and zinc finger nucleases (ZFN), arised the possibility to generate pairs of isogenic lines that facilitate the study of the function of a given gene and the role that different mutations play in the pathophysiological mechanisms of the respective diseases. This approach has been increasingly applied to iPSC lines and could also be very useful in the case of our DPSC-derived cell lines. 1095 1096 1097 1098 1099 1100 1101 1102 1103 1104

Still another naturally-occurring source of SCs are human urine-derived stem cells (USCs), a type of MSCs with proliferation and multi-potent differentiation potential that can be readily obtained from voided urine using an non-invasive protocol and with minimum ethical restriction. These cells express surface markers of MSCs, but not of hematopoietic stem cells, express the stemness-related genes *Nanog* and *Oct3/4* and show telomerase activity, not forming teratomas *in vivo* after being subcutaneously implanted in nude mice [206–209]. When cultured in appropriate media, USCs may differentiate into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic, and neurogenic lineages. Interestingly, USCs may be established from individuals of any age, despite Gao et al. have shown that those isolated from children (5 to 14 year-old) have higher proliferation, lower tendency to senescence, and stronger osteogenic capacity than those from middle-aged (30 to 40 years-old) and elder (65 to 75 year-old) individuals [208]. This property allows to significantly expand the cohort of patients accessible to be studied. Overall, USCs are yet another alternative source of SCs that can be used as a valuable *in vitro* model 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118

to study genetic diseases, with potential applications in regenerative medicine, cell therapy, diagnostic testing and drug screening [210].

## 6. Conclusions

Disease models are essential tools to both identify and study the pathological mechanisms that underlie the development of a disease. They are also a pre-requisite for proper drug development. Indeed, it is essential to have a relevant study model, which reproduces the pathological features of the disease to design and evaluate new therapeutic strategies. And this need goes all the way, from the early *in vitro* assessments to the investigative *in vivo* pre-clinical studies.

Over the last decades, amazing advances have been made on the attempt to model the neuropathology of MPSs *in vitro*, mostly relying on the establishment and subsequent differentiation of disease-specific human iPSCs. And this is certainly true for the larger LSD field, where multiple studies have identified neural progenitor cell migration and differentiation defects, substrate accumulation, axon growth and myelination defects, impaired calcium homeostasis, and altered electrophysiological properties, all using patient-derived iPSCs (reviewed in [211]). So, not even 20 years after iPSCs generation was first described and attempted, their potential to provide mechanistic insights to unravel the pathophysiology associated with neurodevelopment in these rare pathologies is well-established. However, several challenges do remain. That is why we consider it may be useful to contemplate additional sources of patient-derived pluripotent or multipotent cell lines, namely those which are naturally occurring, such as the dental pulp SC derived from human permanent and deciduous teeth. Those cells will also allow for subsequent differentiation into mixed neuronal and glial cultures, which may be analyzed with virtually the exact same methods many authors have been performing to address neuropathology in MPS-derived iPSCs. Finally, regardless of the original source of the SC we are considering, in an era where personalized medicine and mutation-specific therapeutic approaches are gaining momentum, those SC-derived models will also constitute optimal platforms for *in vitro* drug testing.

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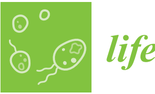

*Review*

# Splicing Modulation as a Promising Therapeutic Strategy for Lysosomal Storage Disorders: The Mucopolysaccharidoses Example

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**Abstract:** Over recent decades, the many functions of RNA have become more evident. This molecule has been recognized not only as a carrier of genetic information, but also as a specific and essential regulator of gene expression. Different RNA species have been identified and novel and exciting roles have been unveiled. Quite remarkably, this explosion of novel RNA classes has increased the possibility for new therapeutic strategies that tap into RNA biology. Most of these drugs use nucleic acid analogues and take advantage of complementary base pairing to either mimic or antagonize the function of RNAs. Among the most successful RNA-based drugs are those that act at the pre-mRNA level to modulate or correct aberrant splicing patterns, which are caused by specific pathogenic variants. This approach is particularly tempting for monogenic disorders with associated splicing defects, especially when they are highly frequent among affected patients worldwide or within a specific population. With more than 600 mutations that cause disease affecting the pre-mRNA splicing process, we consider lysosomal storage diseases (LSDs) to be perfect candidates for this type of approach. Here, we introduce the overall rationale and general mechanisms of splicing modulation approaches and highlight the currently marketed formulations, which have been developed for non-lysosomal genetic disorders. We also extensively reviewed the existing preclinical studies on the potential of this sort of therapeutic strategy to recover aberrant splicing and increase enzyme activity in our diseases of interest: the LSDs. Special attention was paid to a particular subgroup of LSDs: the mucopolysaccharidoses (MPSs). By doing this, we hoped to unveil the unique therapeutic potential of the use of this sort of approach for LSDs as a whole.

**Keywords:** lysosomal storage diseases (LSDs); mucopolysaccharidoses (MPSs); RNA-based therapies; antisense oligonucleotides (ASOs); splice-switching oligonucleotides (SSOs); U1 snRNA (small nuclear RNA)

## 1. Introduction

The somehow recent revolution in RNA biology has led to the recognition of the multiple roles that this molecule may assume within a cell through the identification of new

RNA classes that have previously unanticipated functions. This better understanding of basic RNA biology has been accompanied by a parallel revolution in the use of RNA-based strategies for therapeutic purposes [1]. All of a sudden, RNA-based drugs opened a whole new perspective on therapeutic approaches for previously untreatable diseases by entering the pharmacopoeia and greatly expanding the universe of druggable targets (Figure 1).

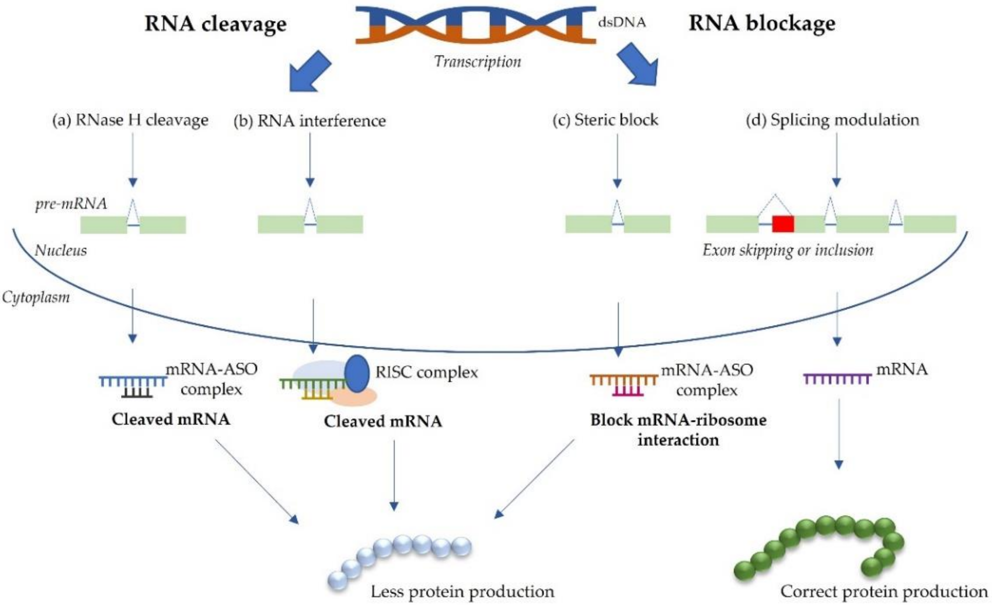


**Figure 1.** Schematic representation of the different mechanisms of action of antisense oligonucleotides (ASOs). ASOs can impact gene expression in different ways, either through RNA cleavage (**a**,**b**) or RNA blockage (**c**,**d**). RNA cleavage (or degradation) approaches include (**a**) RNAse H-mediated mRNA degradation and (**b**) RNA interference (RNAi), while RNA blockage approaches may promote (**c**) sterick block of ribosome binding and (**d**) splicing modulation. The green rectangles represent the coding exonic regions and the blue lines represent the non-coding intronic regions from the pre-mRNA. The red square represents the mutated region of the exon. The dashed lines that form a triangle represent the normal splicing pattern of the pre-mRNA. Abbreviations: ASO, antisense oligonucleotide; mRNA, messenger RNA; pre-mRNA, pre-messenger RNA; RISC, RNA-inducing silencing complex (Adapted from [2]).

Among this promising class of drugs, those that target the splicing process are probably the most widely studied and for which there are five approved drugs for two different diseases [3]. Splicing defects are particularly tempting as therapeutic targets because mutations in the consensus sequences at the borders of introns and exons are a common cause of human genetic diseases. Furthermore, those defects tend to result in the complete loss of function of the protein in question, thus underlying severe pathology [4].

Splicing defects in different genes have been identified as one of the underlying genetic causes of a huge number of genetic diseases of different etiologies. Among those disorders are countless rare diseases of monogenic origin, including the lysosomal storage diseases (LSDs) that were our major focus of interest. LSDs are a particular subset of genetic diseases that can benefit greatly from even the slightest increase in protein function [5]. The vast majority of LSDs are autosomal recessive, even though three X-linked diseases are also known. Still, few disease-specific therapies exist for this vast and heterogeneous group of disorders and even when they do exist, it is now well-recognized that there are some major drawbacks to the existing approaches, such as their inability to act on neurological symptoms [6]. Unfortunately, a great majority of LSDs have a significant neurological component, which is the dominating clinical effect of the disease in a number of disorders, although it is merely one element of a more generalized pathology in others [7]. Among the LSDs that are still lacking effective treatment, a major group is the mucopolysaccharidoses

(MPSs). The MPSs comprise a group of 11 disorders and each one is caused by defects in any of the enzymes that are involved in the stepwise degradation of glycosaminoglycans (GAGs), which lead to the progressive storage of those compounds. This storage, along with other pathogenic mechanisms, triggers several clinical consequences of wide phenotypic variability [8]. Interestingly, even patients that suffer from the same disease can present with extremely different phenotypes that are associated with enzyme activity levels: some patients, who have null or residual enzyme activity, present with early onset severe phenotypes; others, who retain significantly higher residual enzymatic activity, show a much more slowly progressing disorder with a later onset. This means that even a slight recovery in enzyme activity (which can be promoted by the recovery of the normal splicing) can be enough to have a clinical impact [9,10]. Of all MPS-causing mutations, a large percentage affect the pre-mRNA splicing process. Altogether, this makes MPSs excellent candidates for splicing correction therapeutics. Nevertheless, despite the immense potential that these approaches hold for this group of diseases, there are only a few works so far that have attempted splicing modulation approaches for these disorders.

In this work, we address this issue and comment not only on the potential of these drugs but also on the hurdles they must overcome. We start by explaining how splicing can be experimentally modulated for therapeutic purposes. In order to do so properly, we begin by briefly summarizing the normal splicing process and the possible consequences of its disruption. Then, we introduce the currently approved therapeutic approaches that modulate splicing and their mechanisms of action, even though they were not designed for LSDs. Finally, we bring the focus onto our diseases of interest: the MPSs. After an overview of their major clinical features and molecular bases, we outline the contribution of splicing defects to each of the individual diseases. Then, we discuss how some of them have been approached for therapeutic purposes and summarize the published preclinical studies that have assessed the feasibility of recovering pre-mRNA splicing mutations as a way to recover defective enzyme activity. Finally, we comment on the future of splicing therapeutics and the major issues that may hamper their transfer to the clinics and highlight a few strategies that could be used to overcome those hurdles.

## 2. Splicing: How It Works and How It Can Be Modulated

### *2.1. The Splicing Process: Machinery and Mechanisms*

It is well known that eukaryotic gene(s) expression requires a series of highly regulated sequential steps in which non-coding introns are removed from the precursor messenger RNA (mRNA) molecule while the exons, or coding sequences, are joined together, which results in mRNA maturation being translated into protein. This well-known process is called splicing and is carried out by the spliceosome.

RNA splicing was initially discovered in the 1970s and it overturned years of research in the field of gene expression [11,12]. Its major effector, the spliceosome, functions in a complex and dynamic assembly–disassembly cycle in which five small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, U4/U6 and U5) recognize and assemble on each intron to ultimately form a catalytically active spliceosome. An early event in the exon definition is the recognition of the 5′ donor splice site (ss) by the U1 snRNP, which is followed by the binding of splicing factor 1 (SF1) to the branch point and the binding of the U2 auxiliary factor heterodimer (U2AF 65/35) to the polypyrimidine tract (Py) and 3′ss, originating the E complex [13,14]. After that, SF1 is replaced by the U2 snRNP at the branch point, originating the A complex, which allows for the interaction between U1 snRNP and U2 snRNP across the exon [13,15]. Then, the U4, U5 and U6 snRNPs are recruited as a preassembled complex, which leads to the formation of the B complex. Afterward, the interaction between U4 and U6 is disrupted and the U6 snRNP base pairs with the 5′ss, thereby displacing U1 snRNP from its initial location and releasing it from the complex along with the U4 snRNP [16]. At the same time, U6 snRNP interacts extensively with U2 snRNP, which brings the 5′ss and the branch point into close proximity. This allows for the first step of splicing to take place, which originates the C complex, which contains the free

upstream exon and the intron–exon lariat intermediate [15]. This complex completes the second step of the splicing reaction and releases the intron and joins the exons together to form the mature mRNA, while the U2, U5 and U6 snRNPs are also released from the complex and recycled for future splicing reactions [15,17,18].

Although the spliceosome drives pre-mRNA processing with great complexity and fidelity, this is quite a flexible mechanism under the strong regulation by both *cis*- and *trans*-acting elements. The role of *cis*-acting regulatory sequences and RNA-binding protein splicing factors, which recognize and bind to those sites, compose a common mechanism for setting up and maintaining alternative splicing (AS) patterns. Heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine and arginine-rich (SR/AR) proteins in the spliceosome regulate either splicing repression by binding intronic splicing silencers (ISS) and exonic splicing silencers (ESS) or splicing activation by binding intronic splicing enhancers (ISE) and exonic splicing enhancers (ESE) [14,15,19].

AS is a process through which a single precursor mRNA can generate a number of alternative mRNAs, thereby allowing for considerable proteomic diversity and complexity [20,21]. It is currently estimated that nearly 95% of human multi-exonic genes are alternatively spliced, thus giving rise to different protein isoforms. AS mechanisms include: exon skipping, intron retention, mutually exclusive exons and alternative donor 5′ss and acceptor 3′ss [19]. Furthermore, alternative polyadenylation sites and the alternation of the initial exons due to alternative promoter usage can also contribute to AS. In addition, AS can be regulated at the transcription level and in the chromatin structure (Figure 2).

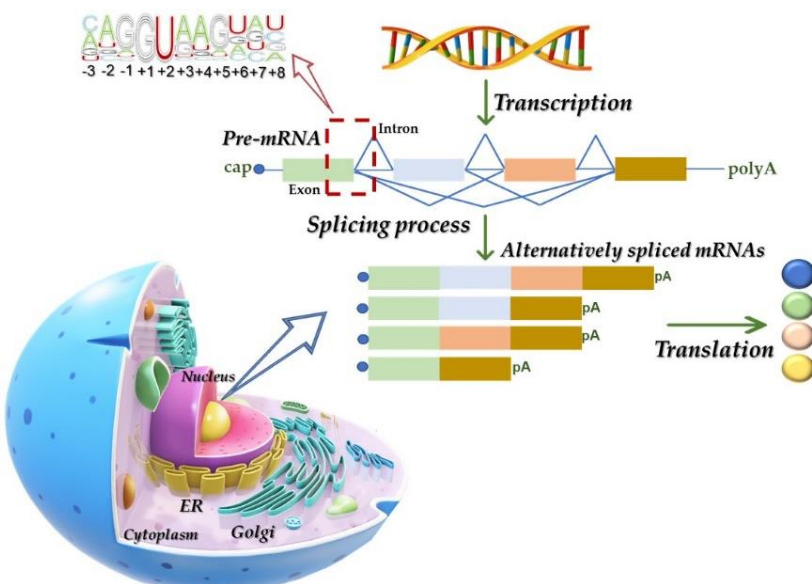


**Figure 2.** Simplified overview of the splicing process. The alternative splicing (AS) process generates mature mRNAs with different exon combinations, which results in the production of different protein isoforms from the same mRNA. Abbreviations: ER, endoplasmatic reticulum; mRNA, messenger RNA; pre-mRNA, precursor mRNA.

A detailed description of the AS process and regulation, which was beyond the scope of this review, can be found in a series of papers that have been published elsewhere [15,22–24]. A variety of therapeutic strategies, such as small molecules and antisense oligonucleotides (ASOs) as well as genome editing through the use of CRISPR/Cas9, have promising future interventions for the amelioration of the disease-causing effects of human mutations on the patterns of AS. Over the following sections, we briefly describe some of the interventions with a special focus on those that are currently approved for commercial use.

*2.2. RNA-Based Approaches for Splice Modulation*

In general, antisense-mediated splicing modulation is a tool that can be exploited in several ways to provide a potential therapy for rare genetic diseases [25]. It is an extremely versatile approach because it can not only promote the correction of cryptic splicing and the modulation of AS, but also the restoration of the open reading frame. Ultimately, it can even induce protein knockdown. This means that splicing modulation approaches can actually go far beyond the correction of individual splicing mutations (such as those that we focus on subsequent sections: see Section 4). Additionally, it may also rely on different effectors, or tools, from antisense oligonucleotides (ASOs) for splicing-switching to synthetic U1 snRNAs (small nuclear RNAs). The most widely known tools that are used to promote splicing correction/modulation are ASOs.

ASOs were first reported by Stephenson and Zamecnik in 1978 [26]. ASOs are short synthetic oligonucleotides (15–30 nucleic acid length) designed complementary to sense strand of mRNA and efficient laboratory tools that can regulate the expression of specific genes through an efficient modulation of the splicing process [27]. When designed to target the splice site or its auxiliary sequences, which leads to mRNA repair and the restoration of protein function and modifies the outcome of the splicing reaction, they are called splice-switching ASOs or splice-switching oligonucleotides (SSOs). These ASOs are able to sterically block relevant motifs in the pre-mRNA without promoting its degradation.

Numerous studies have investigated the therapeutic potential of ASOs in *in vitro* cell models, animal disease models and human clinical trials. Even though a complete overview of all of these studies clearly fell outside of the scope of this review, we briefly discuss the approved therapeutic strategies to treat diseases using ASOs. By doing so, we hope to unveil the full potential of this somewhat novel class of drug and show how life-changing these molecules can be for patients who harbor different genetic mutations, provided that a number of requirements are met.

The demonstration that an ASO drug can successfully promote the correction of its targets *in vivo* paved the way for the clinical trials of ASOs as a treatment for a variety of diseases, especially rare diseases such as Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA). Currently, there are a number of approved drugs for these pathologies, all of which are capable of manipulating the pre-mRNA splicing process: Eteplirsen (EXONDYS 51™, Sarepta Therapeutics, Cambridge, MA, USA) [28,29]; Golodirsen (Vyondys 53™, Sarepta Therapeutics, Cambridge, MA, USA) [30]; Viltolarsen (Viltepso®, NS Pharma, Paramus, NJ, USA) [31]; and Casimersen (Amondys 45™, Sarepta Therapeutics, Cambridge, MA, USA) [32] for the DMD and Nusinersen (Spinraza®, Biogen, Cambridge, MA, USA) [33,34] for SMA (Table 1).

DMD is an X-linked genetic disease that is characterized by the absence of the dystrophin protein in muscle fibers, which is manifested by progressive muscle degeneration and weakness. Approximately two thirds of DMD cases present deletion mutations in the *DMD* gene, which is composed of 79 exons (the largest known human gene) [35]. Becker muscular dystrophy (BMD) is a mild disease that is caused by dystrophin truncations and not by its absence. To produce mild phenotypes, such as BMD, a strategy that can generate a truncated but functional dystrophin protein would be a reliable tool. Thus, the skipping of exons to correct DMD-linked mutations can reduce the severity of the disease and produce a phenotype that is similar to that of BMD [36]. Eteplirsen, which is a 30-nucleotide phosphorodiamidate morpholino oligomer (PMO), binds to the 5′ss of exon 51, which leads to it being skipped (Figure 3b). Thus, an in-frame transcript is produced that allows for the formation of an internally truncated but functional dystrophin protein [36,37]. Eteplirsen can only be used for patients who are amenable to exon 51 skipping, which accounts for 13% of the DMD patient population [38]. In September 2016, this drug received approval from the US Food and Drug Administration (FDA), which made it the first ASO to be approved for DMD and the first approved exon skipping ASO to be used for humans [38].

**Table 1.** Antisense oligonucleotides (ASOs) that are approved for Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA) treatment.

| Brand Name | Drug | Year of Approval | Target Molecule | Treatment Result | Target Disease |
|---|---|---|---|---|---|
| Spinraza®, Biogen | Nusinersen | 2016 | *SMN2* mRNA | Induces the inclusion of exon 7 in the *SMN2* mRNA | Spinal muscular atrophy |
| Exondys 51™, Sarepta Therapeutics | Eteplirsen | 2016 | Dystrophin mRNA | Induces the exclusion of exon 51 of dystrophin mRNA | Duchenne muscular dystrophy |
| Vyondys 53™, Sarepta Therapeutics | Golodirsen | 2019 | Dystrophin mRNA | Induces the exclusion of exon 53 of dystrophin mRNA | Duchenne muscular dystrophy |
| Viltepso®, NS Pharma | Viltolarsen | 2020 | Dystrophin mRNA | Induces the exclusion of exon 53 of dystrophin mRNA | Duchenne muscular dystrophy |
| Amondys 45™, Sarepta Therapeutics | Casimersen | 2021 | Dystrophin mRNA | Induces the exclusion of exon 45 of dystrophin mRNA | Duchenne muscular dystrophy |

More recently, in 2019, another ASO drug was approved to treat this disease: Golodirsen. This is a 25-mer PMO that binds to the exon 53 of the *DMD* gene and causes it to be skipped, thereby avoiding the deleterious loss-of-function frameshifting mutations [30,39]. It was only approved for males with mutations that are amenable to exon 53 skipping. Then, in 2020, yet another drug for the treatment of DMD patients with the same characteristics was approved by the FDA: Viltolarsen, which is a 21-mer PMO that also binds to exon 53 and causes it to be skipped [31,40] (Figure 3c). In both cases, the skipping of this exon restores the reading frame and leads to the production of an internally truncated but partially functional dystrophin protein [41]. Both drugs are suitable for 8% of DMD patients. Finally, in 2021, an ASO from the PMO subclass was developed by Sarepta Therapeutics for the treatment of DMD in patients who have a mutation of the *DMD* gene that is amenable to exon 45 skipping: Casimersen. Casimersen was designed to bind to the exon 45 of the *DMD* gene pre-mRNA and leads to the production of an internally truncated but functional dystrophin protein [32] (Figure 3d).

Altogether, ASOs that address the primary genetic defect of DMD are among the first generation of therapies tailored to overcome specific genetic mutations in humans. They represent paradigm-forming approaches to medicine that may lead to life-changing treatments for those affected by this relentlessly progressive and fatal disease [42].

SMA is another disorder that has greatly benefited from the development of splice modulation therapeutics. SMA is an autosomal recessive neuromuscular disease that is caused by mutations and deletions in the survival motor neuron 1 (*SMN1*) gene, which results in the progressive loss of alpha motor neurons in the anterior horn of the spinal cord [43]. A second *SMN* gene exists in human genome: the *SMN2* that has a C to T mutation in exon 7. This single nucleotide change does not affect the protein sequence but it does affect the pre-mRNA splicing, which gives rise to an unstable isoform that is rapidly degraded [44,45] (Figure 4a).

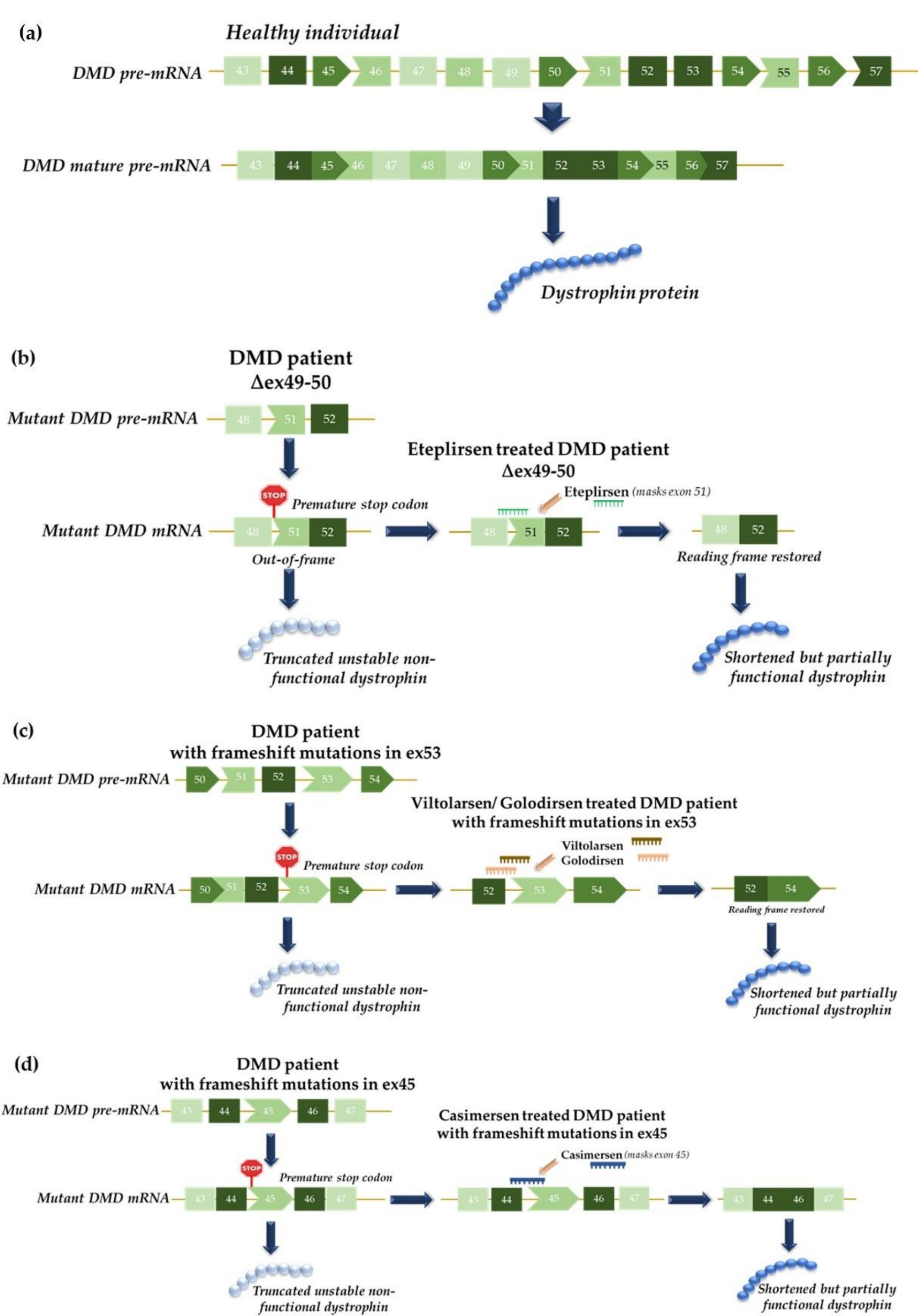


**Figure 3.** Mechanism of action of exon skipping therapy for Duchenne muscular dystrophy (DMD): (**a**) schematic representation of the normal splicing of the *DMD* gene in healthy individuals who produce normal dystrophin protein. In general, treatment of DMD with antisense oligonucleotides (ASOs) promotes selective exon skipping in order to restore the reading frame and produce a truncated but partly functional dystrophin protein. Different drugs are available for the different mutations that affect a number of *DMD* exons: (**b**) Eteplirsen, for DMD patients with deletions spanning exons 49 and 50; (**c**) Viltolarsen/Golodirsen, for DMD patients with frameshift mutations in exon 53; and (**d**) Casimersen, for DMD patients with frameshift mutations in exon 45. Abbreviations: Δ49-50, deletion of exons 49 and 50; DMD, Duchenne muscular dystrophy; mRNA, messenger RNA; pre-mRNA, precursor mRNA.

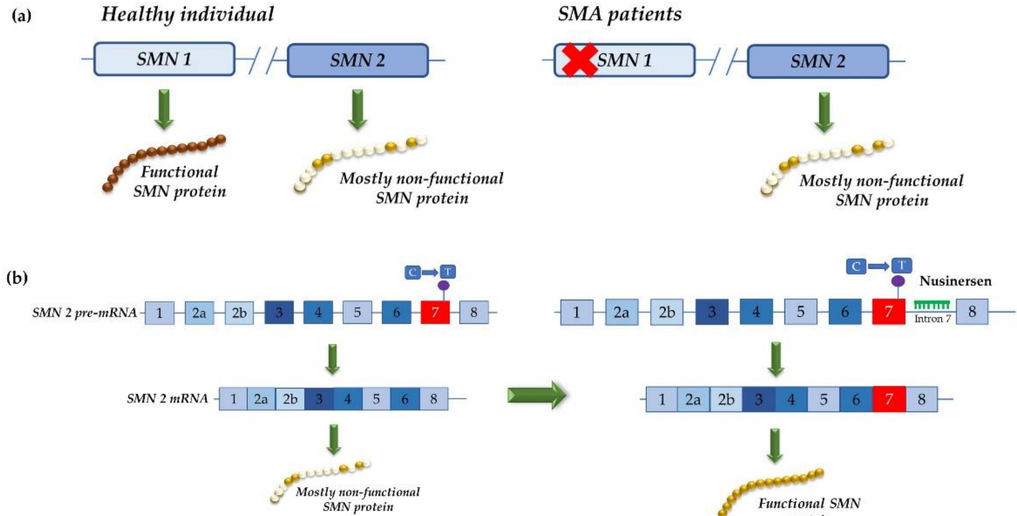


**Figure 4.** Mechanism of action of exon inclusion therapy for spinal muscular atrophy (SMA): (**a**) overview of the molecular basis of SMA. Humans have two *SMN* genes: *SMN1*, which gives rise to a functional SMN protein and *SMN2*, which has a C to T mutation in exon 7 that does not affect the protein sequence but does affect the pre-mRNA splicing, thereby giving rise to an unstable isoform that is rapidly degraded. In healthy individuals, the presence of a functional SMN protein that is encoded by the *SMN1* gene assures the assembly of the cellular machinery that is needed to process pre-mRNA. In SMA patients, mutations in the *SMN1* gene prevent the production of a functional SMN protein: (**b**) Nusinersen targets and blocks the intronic splicing silencer (ISS) in intron 7, which induces the inclusion of exon 7 in the *SMN2* mRNA. Abbreviations: C, cytosine; mRNA, messenger RNA; pre-mRNA, precursor mRNA; SMA, spinal muscular atrophy; SMN, survival motor neuron; T, thymine.

Taking this into account, Cartegni and colleagues showed that a 2′-O-methoxyethyl (2′MOE) phosphorothioate-modified ASO can efficiently correct *SMN2* exon 7 splicing both *in vitro* and *in vivo* [43,46,47]. By targeting and blocking the intron 7 ISS, Nusinersen induces the inclusion of exon 7 in the *SMN2* mRNA. This ASO was approved by the FDA in December 2016 [48] (Figure 4b). Together with the two other approved drugs for SMA replacement therapy, Nusinersen has provided a life-changing treatment option for SMA patients and their families. It extends life expectancy and allows patients to reach motor milestones that would previously have been unachievable [49].

The second major approach for the modulation of the splicing process, both *in vitro* and *in vivo*, is the use of synthetic U1 snRNAs designed to recognize mutant 5′ss, thus restoring complementarity. The first step of the splicing process requires the 5′ end of the U1 snRNA to interact by complementarity with the moderately conserved sequence of the 5′ss [20]. This implies that any mutation in this site may compromise the binding of the U1 snRNA and prevent spliceosome assembly, thus inhibiting the subsequent splicing process. Therefore, these sorts of variants usually cause disease.

Over the last decade, U1 snRNAs with a modified 5′ tail that base pair exactly to the mutant splice site have been used to correct 5′ss mutations, abolishing the skipping of some the exons that they originally caused. Another type of modified U1 snRNAs are the so-called exon-specific U1 snRNAs (ExSpe U1s), which have also been tested in different *in vitro* and *in vivo* approaches that have shown their therapeutic potential [20,50–52]. Recently, Balestra and colleagues published the *in vivo* proof of principle for the correction potential of compensatory U1 snRNAs in hereditary tyrosinemia type I [50]. Nevertheless, this approach is not yet available as a therapeutic option and more studies are needed before its translation into the clinic.

The combined use of ASOs and U1snRNAs is also under consideration. In fact, a combined treatment using ASOs and engineered U1 snRNAs has shown the highest

therapeutic efficacy for correcting mutation-induced splicing defects in Bardet–Biedl syndrome [51]. This recent observation has shown that there may be an advantage in the use of these two therapeutic approaches with complementary effects for the improvement of treatment efficacies.

Among the monogenic diseases, which may benefit from either sort of splice-modulation therapeutics are the LSDs, a group of life-threatening disorders, which are further addressed in the following sections.

### *2.3. Hurdles*

Despite its promise, the development of RNA therapeutics has faced several major hurdles over recent decades, namely: (1) the rapid degradation of exogenous RNAs by ubiquitous endogenous RNases; (2) the challenging delivery of negatively charged RNA molecules across hydrophobic membranes; and (3) the strong immunogenicity of synthetic RNAs, which ends up causing cell toxicity and impairing translation. These hurdles have been substantially overcome with recent advancements in RNA biology, bioinformatics, separation science and nanotechnology, all of which have greatly facilitated the recent rapid development of RNA therapeutics as a whole [53].

However, there are several challenges that may still hinder the prompt clinical translation of some RNA drugs. Most of these challenges are common to all types of RNA drugs, but others are specific to those that are aimed at splicing modulation.

For example, the development of proper models to assess the sequence-dependent efficacy and safety of ASOs is still a pending issue [54]. This is particularly relevant for the splicing modulation approaches designed to correct specific disease-causing mutations that affect the normal splicing process, the so-called splicing mutations. Ideally, the preclinical development of that sort of drugs would require the development of animal models that carry the specific splicing mutations. Importantly, however, an alternative exists for a few specific approaches that does not require these mutation-specific models. In fact, for the therapies that rely on the promotion of the skipping of a specific exon, it is possible to use wild animals instead of mutation-specific models.

Then, there is the question of the species-specific sequence differences between orthologous genes. SSOs and U1snRNA-based therapies are sequence-specific approaches that aim to interfere with the splicing mechanism and they are specifically designed to recognize a certain target sequence in the human genome. Unfortunately, most of our sequences of interest are not completely conserved among different species. Therefore, the molecules designed to target a human sequence cannot be directly assessed in an animal model [55]. This means that for *in vivo* assessments, it is not usually possible to use exactly the same SSOs or U1snRNA sequences that are used for human cells. It is always necessary to design species-specific SSOs and U1snRNAs (i.e., specifically designed for animal sequences, which are orthologous to the human genes under study). This is actually the standard approach for *in vivo* ASO studies and most of the currently approved ASOs relied on the *in vivo* assessment of animal responses to slightly modified molecules, which were designed to match the orthologue sequences. This sort of *in vivo* studies may provide relevant safety and toxicity data, but it relies on the premise that the consensus splice site sequences in mice and humans are highly conserved and comparable. Still, some small changes in these patterns have been described [56].

The alternative would be to generate humanized animal models, an approach that is both time- and resource-consuming and may contribute to a substantial increase in the drug development time while requiring additional funding. Furthermore, the generation of a humanized animal model for every mutation that needs to be targeted is neither feasible nor ethical and may not always recapitulate the human molecular and/or physiological phenotypes [54,55].

The last and probably the major challenge that could hinder the broader clinical translation of this category of drugs is their inefficient delivery to the target tissues. This is not only true for splicing modulation but also for every other RNA-based approach. In general,

the delivery of ASOs and any other RNA-based drugs to target tissues is relatively poor after systemic delivery. Nevertheless, relevant increases in the efficiency of ASO delivery have been achieved over recent years through chemical modification and conjugation to other moieties, as well as the development of new chemical backbones. Furthermore, many teams have been working on the development of effective drug delivery systems, which ultimately enhance the delivery of drugs to the target sites of pharmacological action. Among these systems, lipid nanoparticles (LNPs) and/or adeno-associated viruses (AAVs) are probably the most well known (reviewed in [57]). Nevertheless, the latest advances in ASO technology have been coupled with the surprising finding that despite being highly charged and large, ASOs distribute widely throughout the CNS when they are delivered to the cerebral spinal fluid via intrathecal (IT) delivery, which is safe and well tolerated. This peculiarity (contrary to other RNA therapies, such as siRNAs and U1snRNAs vectors) has greatly enabled the application of ASOs as a therapeutic strategy for CNS disorders, many of which currently have no treatment [58]. Remarkably, IT ASO administration has already been implemented for the treatment of SMA and has produced safe and tolerable results [58,59]. An ASO that targets ALS and is delivered via IT was also recently administered to one patient [60].

Over the last decade, huge successes have also been documented for therapies that target hepatocytes and in which GalNAc conjugation and LNP technology allow for the targeted delivery of drugs with outstanding results, which has resulted in approval being granted for several clinical indications. These examples of how specific and well-designed drug delivery technologies can be used to overcome the targeting hurdles have provided a new impetus to the RNA-based therapeutics field, which will certainly contribute to fostering research and accelerating discoveries about extra-hepatic delivery (reviewed in [61]). Another drawback is the high exposure of certain organs upon the systemic delivery of AONs. For instance, after the intravenous injection of AONs, a significant proportion is absorbed by the liver and kidneys. This limits their biodistribution to other tissues and results in a toxic effect within these organs. Importantly, however, many of the liver and kidney injuries were found when using high and not clinically relevant doses of AONs. Obviously, the design and manufacture of efficient delivery systems is not the only hurdle. Their safety, both alone and in combination with RNA-based drugs, is also paramount [61].

## 3. Treatment Strategies for LSD Patients: MPSs in the Spotlight

### *3.1. Lysosomal Storage Diseases*

Lysosomal storage diseases (LSDs) are a group of about 70 monogenic and hereditary diseases of lysosomal catabolism. The majority of them are inherited in an autosomal recessive manner, but three diseases are X-linked. These disorders have a combined incidence of around 1:7700 but, according to several authors, this figure may be as high as 1:3000 or even 1:1500 when all LSDs are considered [62,63]. LSDs occur when a mutation, or more than one mutation, occurs in genes that code for proteins that are important for lysosomal function (i.e., lysosomal proteins, in the majority cases), thus affecting their function. This results in lysosomal malfunction and the gradual storage of the undegraded/partially degraded substrates inside the lysosome, which ultimately results in cell dysfunction and death [64,65].

Frequently, LSDs present as pediatric neurodegenerative diseases [66]. However, as they are heterogeneous disorders, depending on the gene defect and on the biochemical nature of the stored substrates, lysosomal storage defects can cause skeletal dysmorphia, due to bone pathology, and central nervous sys-tem (CNS) defects, in addition to symptoms affecting many other organs..

LSD diagnosis is usually based on the clinical symptoms of patients, followed by the confirmation of increased storage and genetic alterations through several diagnostic tests, such as enzymatic analysis and gene sequencing. More recently, diagnosis through next

generation sequencing (NGS) has become routine, which greatly reduces the time from the initial presentation of symptoms to the diagnosis of the disease [67,68].

Based on the type of disorder and the age of diagnosis, LSDs can be classified into congenital or infantile, late-infantile, juvenile and adult types. Usually, the earlier the symptoms appear, the more severe the disease presentation.

Treatment strategies for LSDs include: enzyme replacement therapy (ERT), which consists of providing the missing/defective enzyme; substrate reducing therapy (SRT), in which the synthesis of the accumulated substrates is reduced; hematopoietic stem cell transplantation (HSCT), in which healthy matched donor cells are transplanted into the patient and the enzyme is then secreted continuously from the donor cells; and chaperone therapy, which encompasses the use of competitive inhibitors at sub-inhibitory concentrations to stabilize the mutant enzyme, thereby extending the half-life and improving catalysis. Even though treatments are available for 11 LSDs, most of these disorders are managed symptomatically and patients only receive supportive care due to the inability to treat neurological symptoms [64].

Most importantly, even when therapies are available, especially ERTs, they are only successful in the somatic tissues of the body and cannot cross the blood–brain barrier (BBB); therefore, they fail to treat neurological deficits, which are among the most debilitating symptoms of many LSDs. Once neurological damage has occurred, it is extremely difficult to revert the phenotype. Thus, obtaining the correct enzyme dose in the brain is a major therapeutic goal. About two thirds of LSDs have neurological involvement [59]. This is why small-molecule drugs are being developed to cross the BBB, even though, so far, none reliably reach the brain. However, gene therapies that directly target the CNS are promising.

### 3.2. *Mucopolysaccharidoses*

One of the subgroups in which neurological symptoms are the most prevalent is the mucopolysaccharidoses (MPSs), which represent approximately 30% of LSD cases [69]. Seven major MPSs are currently known (MPSI, II, III, IV, VI, VII and IX), which result from mutations in the genes that code for one of 11 acid hydrolases involved in the degradation of GAGs. Each individual enzyme deficiency underlies one particular MPS (for instance, four different deficiencies trigger an equivalent number of MPS III disorders) [70] (Table 2). As these lysosomal enzymes fail to fulfill their function, the compounds accumulate in cells and tissues, which then causes progressive damage and a variety of clinical multi-organ manifestations, such as cardiovascular disease, respiratory problems, skeletal abnormalities and premature death, but the spectrum and severity of the disease manifestations vary between and within the MPS types [8,71]. These compounds can also accumulate outside of the lysosomes, thereby activating inflammatory pathways and an innate immune response via the tool-like receptor 4 and the complement system. Aspects such as neuroinflammation, short bones and aortic fragmentation can also arise due to this inflammatory response [8].

MPSs are heterogeneous and multisystemic diseases and manifestations vary not only between the subtypes but also within the same subtype. These characteristics affect the quality of life and lifespan of the patients. Clinically, MPS patients can be classified as having a “visceral phenotype”, a “neurodegenerative phenotype” or a “skeletal phenotype”, depending on the subtype of the disease. In general, MPS types I, II, VI and VII present with coarse facies, visceromegaly (hepatosplenomegaly), hernia, upper airway obstruction, joint stiffness, heart disease and other skeletal deformities as the main group characteristics. Due to these manifestations, these MPSs are usually classified as the group with “visceral phenotype”. A short stature is present in MPS I, II and VII patients. Furthermore, corneal clouding is also very frequent in all of these subtypes, except for type II, in which hearing loss is marked [8]. MPS III patients belong to the group with “neurodegenerative phenotype”, in which the clinical manifestations of the groups that were referred to above are mild but there is a marked neurodegeneration, which usually starts between 3 and 5 years of age and is accompanied by behavioral disturbances and hyperactivity. Finally, the “skeletal phenotype” is a characteristic of MPS IV patients, who show skeletal dysplasia

and many other bone problems. They are mentally normal and have a short stature. MPS IX is not included in these three groups because the main clinical manifestation is the presence of joint swelling and synovial masses [8].

**Table 2.** Classification of mucopolysaccharidose (MPS) subtypes.

| MPS Type | Common Name(s) | Associated Gene | Enzyme Deficiency | Number of Mutations | % of Splicing Mutations | Treatment Options Available |
|---|---|---|---|---|---|---|
| I | Hurler, Scheie and Hurler–Scheie syndromes | *IDUA* | Alpha-L-iduronidase | 320 | 15.3 | ERT, HSCT |
| II | Hunter syndrome | *IDS* | Iduronate-2-sulfatase | 739 | 8.8 | ERT, HSCT |
| IIIA | Sanfilippo syndrome type A | *SGSH* | Heparan-*N*-sulfatase | 163 | 2.5 | - |
| IIIB | Sanfilippo syndrome type B | *NAGLU* | *N*-acetylglucosaminidase | 256 | 3.1 | - |
| IIIC | Sanfilippo syndrome type C | *HGSNAT* | Acetyl CoA glucosamine *N*-acetyltransferase | 91 | 17.6 | - |
| IIID | Sanfilippo syndrome type D | *GNS* | *N*-acetyl-glucosamine-6-sulfatase | 28 | 14.3 | - |
| IVA | Morquio syndrome type A | *GALNS* | *N*-acetylgalactosamine-6-sulfate sulfatase | 378 | 10.3 | ERT, HSCT |
| IVB | Morquio syndrome type B | *GLB1* | β -galactosidase | 265 | 8.3 | - |
| VI | Maroteaux–Lamy syndrome | *ARSB* | Arylsulfatase B | 229 | 5.7 | ERT |
| VII | Sly syndrome | *GUSB* | β-glucuronidase | 81 | 7.4 | ERT |
| IX | Hyaluronidasedeficiency | *HYAL1* | Hyaluronidase | 7 | 0 | - |

All subtypes are monogenic diseases that are transmitted in an autosomal recessive way except for MPS II, which is X-linked. In general, nonsense and frameshift mutations seem to lead to a more severe disease, while missense mutations are associated with more attenuated forms. Splicing mutations are generally associated with severe disease forms, but when the normal transcript is produced (even in small amounts), a milder phenotype can be present. This genotype–phenotype correlation can help to predict phenotype, which is very important for MPS I patients, for example, to ensure that the correct treatment option is applied. However, it is difficult to predict that the phenotype on an individual basis. This is why it is important to study the impact of each mutation at the cDNA and protein level, as well as develop new biomarkers for the assessment and follow-up of treated and untreated patients [72,73].

MPS type I is the most frequent form of MPS and results from mutations in the *IDUA* gene that codes for α-L-iduronidase (EC 3.2.1.76). A deficiency of this enzyme results in the lack of the degradation of dermatan and heparan sulphates (DS/ HS), which leads to their progressive accumulation. A wide range of phenotypic involvement exists, including three major recognized clinical entities: Hurler (MPS IH; OMIM #607014), which is the most severe; Scheie (MPS IS; OMIM #607016), which is milder; and Hurler–Scheie (MPS IH/S; OMIM #607015), which has an intermediate phenotype [74]. The incidence of MPS I is estimated to be approximately 1:100,000 births (reviewed in [73]). To date, at least 320 mutations in *IDUA* are known, of which 15.3% are splicing mutations ([75]; Table 2). The early initiation of treatment, as for all treatable LSDs, results in more favorable outcomes. For this subtype, treatment options include HSCT, which is the gold standard for severe forms of the disease and for young children in the early stages of Hurler syndrome, and ERT with recombinant laronidase (Aldurazyme®, Genzyme), either alone or in combination [73,76–78]. However, the diagnosis of MPS I is often difficult, particularly for patients with attenuated phenotypes, which results in the delayed introduction of treatment. Gene therapy for MPS I is still only in the preclinical stages of development [77].

MPS II, also known as Hunter syndrome (OMIM #309900), is caused by mutations in *IDS* gene, which result in a deficiency of iduronate-2-sulfatase activity (EC 3.1.6.13). This decreased activity leads to intracellular and extracellular accumulation of HS and DS in various organ systems, as in MPS I. This disease is the only MPS that is not inherited in an autosomal recessive manner but rather has an X-linked inheritance [73,79]. So far, at least 739 mutations in *IDS* are known, of which 8.8% are splicing mutations ([75]; Table 2). These genetic variations result in different phenotypes of the disease, which can be classified as severe or attenuated. The severe form affects about 60% of patients and has CNS involvement. The overall estimated incidence of MPS II is 1:162,000 live male births [79].

The two approved treatments for MPS II are ERT with recombinant human IDS infusions of idursulfase (Elaprase®, Shire) and HSCT, which has been shown to have neurological benefits in MPS II patients.

Sanfilippo syndrome, or MPS III, can be differentiated from the other types due to the predominance of CNS disease [59,80]. The main compound that is accumulated is HS. Depending on the mutated gene and, consequently, the associated enzyme deficiency, this type can be classified as: MPS IIIA (OMIM #252900), with mutations in the *SGSH* gene; IIIB (OMIM #252920), when the mutations are in *NAGLU*; IIIC (OMIM #252930), which is caused by mutations in the *HGSNAT* gene; or IIID (OMIM #252940), with mutations in *GNS*. To date, numerous mutations have been identified in each of the four genes, 2.5%, 3.1%, 17.6% and 14.3% of which affect the splicing process for subtypes A, B, C and D, respectively ([75]; Table 2). Somatic symptoms are mild, even though hepatosplenomegaly is often present but not usually diagnosed clinically, and cardiac problems are rare (reviewed in [81]). As HS accumulates primarily in the brain, classical ERT, which is the most successful strategy for other non-neurological LSDs, may not be effective. The BBB limits the availability of the enzyme in the brain and IT and intracerebroventricular (ICV) administrations are very invasive strategies that have a number of associated problems. Clinical trials have been conducted to investigate various methods for ERT delivery to the CNS; however, they have been shown not to promote neurocognitive benefits [82–84]. A recent clinical trial of MPS IIIA patients using IT administration for the defective enzyme showed a reduction in HS and GAG levels in the treated patients. Still, the primary neurocognitive endpoint was not met [83]. Currently, there are no available treatments for this syndrome. Most efforts are palliative and focus on regulating behavior (aggressiveness, hyperactivity, etc.) and sleep disturbances. However, a number of therapies are now being developed and evaluated, such as HSCT, gene therapy, SRT and anti-inflammatory therapies (reviewed in [80,85]).

MPS IV, also known as Morquio syndrome, is caused by the impaired degradation of keratan sulphate (KS). Two enzyme deficiencies are known to lead to this syndrome: N-acetylgalactosamine-6-sulphatase (GALNS; EC 3.1.6.4), which causes Morquio syndrome type A (OMIM #253000), and β-galactosidase (EC 3.2.1.23), which causes Morquio syndrome type B (OMIM #253010). To date, at least 378 mutations are known for MPS IVA, of which 10.3% are splicing mutations, and 265 are known for MPS IVB, 8.3% of which are known to affect the splicing process ([75]; Table 2). Both forms of MPS IV have skeletal dysplasia, very short stature, ligamentous laxity/joint hypermobility and odontoid hypoplasia as major characteristics. Most patients are mentally normal [70,86]. Nevertheless, neurological involvement can also occur in severe cases and can be life-threatening, with the affected individuals not normally surviving past the second or third decade of life. Those patients with milder forms of the disorder usually survive to adulthood, even though their life expectancy may be reduced [8]. ERT using recombinant human GALNS, elosulfase alfa (Vimizim®; BioMarin Pharmaceutical Inc) and HSCT are the treatment options for MPS IVA (reviewed in [87]). There are no therapies currently available for MPS IVB.

MPS type VI, or Maroteaux–Lamy syndrome (OMIM #253200), results from a deficiency of arylsulfatase B (N-acetylgalactosamine-4-sulfatase; EC 3.1.6.12), which is caused by mutations in the *ARSB* gene. This deficit results in the pathological accumulation of DS in most organs and systems. The incidence estimates range from 1:77,000 to 1:278,000 live births. Presently, 229 mutations in *ARSB* are known, of which 5.7% affect the normal

splicing process ([75]; Table 2). As with MPS IV, a purely somatic disease occurs with no cognitive involvement. Patients present within a spectrum of clinical severity: when they have a severe case of the disease, i.e., showing the onset of symptoms before 2 or 3 years of age and impaired mobility by 10 years of age, usually die in second or third decade of life; when the disease is attenuated, patients have a later onset of symptoms and tend to be diagnosed either in their teens or in early adulthood [88]. ERT with galsulfase (Naglazyme®, BioMarin Pharmaceutical Inc) is currently the recommended first-line treatment for MPS VI, although there have been various studies published on the positive effects of HSCT and the combination of the two treatments on MPS VI patients (reviewed in [89]).

MPS VII, also known as Sly syndrome (OMIM #253220), is a rare type of MPS that is characterized by the lack of the β-D-glucuronidase enzyme (EC 3.2.1.31) due to mutations in the *GUSB* gene. This deficiency causes an accumulation of DS, HS and chondroitin sulphate (CS) proteoglycans, which are mainly sulfated in the 4 (C4S) and 6 (C6S) positions, in multiple tissues. MPS VII patients are phenotypically heterogeneous but there are a few common features that can be recognized, including short stature, coarse facial features, corneal clouding, hydrocephalus, skeletal deformation and cardiac diseases, similar to those features that are observed in MPS I and II. Interestingly, a distinguishing feature is observed in this subtype: hydrops fetalis, which is an abnormal accumulation of bodily fluids in several tissues [90,91]. To date, 81 mutations have been identified in *GUSB*, 7.4% of which are splicing mutations ([75]; Table 2). For the non-neurological manifestations of MPS VII, ERT with vestronidase alfa (Mepsevii™, Ultragenyx, Novato, CA, USA), which was approved by the FDA in 2017, is the recommended therapeutic approach [92]. As for the other types of MPS, HSCT has also been studied in MPS VII patients but no definitive conclusions about its therapeutic efficacy have yet been drawn due to the limited data (reviewed in [93]).

Finally, MPS IX, also known as hyaluronidase (EC 3.2.1.35) deficiency (OMIM # 601492), is caused by mutations in the *HYAL1* gene, which results in the accumulation of hyaluronan. It is an ultra-rare type of MPS and, to date, only four patients have been reported worldwide: one patient in the original report was diagnosed in 1996 and the three other patients belonged to a second family, who were diagnosed in 2011 [94,95]. All reported patients with MPS IX presented with joint and skeletal problems. According to the data that were collected from these patients, there are only seven mutations that are known to be responsible for this disease, none of which affect the splicing process ([75], Table 2).

Altogether, excluding the ultra-rare MPS IX, which has no associated splicing defects, 3% to 18% of the currently described mutations are known to disrupt the normal pre-mRNA splicing, depending on the MPS type being considered. This reinforces the need for a deeper study on the effects of this type of mutation, but it also makes them great candidates for splice modulation approaches. While 11 different MPSs exist and only 5 of them have approved therapeutic approaches, the need for additional treatment options is real. It is also worth mentioning that, even for the diseases that do have treatments available, the currently approved drugs fail to address CNS lesions, thus allowing for the neuropathological progression of the disorder and the resultant neuropsychiatric manifestations [96]. In fact, the development and delivery of effective treatments for these neurological and psychiatric signs and symptoms are universal hurdles that are faced not only by MPSs, but also by virtually every other LSD. This is why so many different therapeutic approaches are either being developed or are under evaluation for this group of disorders, from substrate reduction to gene therapy [97]. Also included among those possibilities are patient-tailored, mutation-specific approaches, which take advantage of the current knowledge on the molecular basis of these disorders to design a drug which holds potential to surpass the molecular defect that underlies pathology in one particular patient. Ultimately, there is even room for the so-called N-of-1 therapeutics, in which a drug is specifically designed for the treatment of just one patient.

## 4. RNA-Based Therapeutic Approaches for MPS Mutations

Altogether, there are at least 226 MPS-causing mutations that affect the pre-mRNA splicing process [75]. These mutations can occur in *cis*-acting elements, including 5′ss and 3′ss, GU-AG canonical nucleotides, the Py tract, branch point sequence, ESE, ESS, ISE and ISS, which affects their interaction with *trans*-acting factors (SR family proteins and hnRNPs). These mutations can have a higher frequency worldwide, can be identified in a small number of families or they can be unique. While some notable exceptions have been recognized for a few LSDs [9], no MPS-related splicing mutations have yet been identified as being particularly prevalent among affected individuals and/or specific populations. Nevertheless, MPS-causing mutations are good candidates for splicing modulation approaches for several reasons, which we have already listed. Over the following sections, we summarize functional studies that have focused on MPS-causing mutations that affect splicing, as well as the studies that we are aware of that have attempted the correction and/or amelioration of MPS disease phenotypes through splice modulation.

### *4.1. Functional Studies of Splicing Mutations and Development of Therapeutic Approaches Using Antisense Oligonucleotides: The MPS II Example*

So far, 739 MPS II causal mutations have been reported in the *IDS* gene (OMIM *309900), 65 of which have been described as affecting splicing (around 8.8%) [75]. In a study that was published in 2015, Matos et al. performed an extensive functional analysis on three *IDS* gene splicing mutations in order to better understand how and why splicing is altered and they subsequently addressed the *in vitro* correction of one of them using splicing-related ASOs [98,99]. Two of them, c.257 C>T and c.241 C>T, are located in exon 3 and activate a cryptic splice site in this exon. The third, c.1122 C>T, is located in exon 8 of *IDS* and is responsible for the creation of a new 5′ss, which leads to a shorter transcript than wild-type.

This is particularly relevant since only two of these disease-causing variants had previously been characterized at cDNA level and shown to disrupt the normal *IDS* splicing process: c.257 C>T and c.1122 C>T. The third, while previously reported, had only been analyzed at the gDNA level and incorrectly classified as a nonsense mutation [100]. Reporter minigenes were used as tools to perform these functional analyses. In fact, there is a significant number of papers on the efficacy of *in silico* predictors, which directly compare the bioinformatic results to those that were obtained with reporter minigenes, taking the latter as "controls", and only analyze patient RNA when available [101]. This is why the effects of intronic or exonic mutations on splicing should ideally be assessed both by *in silico* tools and through the construction and transient expression of minigenes that harbor the variants under analysis.

Moreover, the splicing regulation of exon 3 has also been addressed using mutant minigene analysis and overexpression/silencing assays. It was observed that SRSF2 and hnRNP E1 could be involved in the use and repression of the constitutive 3′ss of exon 3, respectively [98]. These two regulatory elements, SRSF2 and hnRNP E1, were overexpressed or silenced in the Hep3B cell line that was transfected with either wild-type (WT) or mutant minigenes. It was verified that the choice of the constitutive 3′ss of *IDS* may be dependent on an ESE site that is recognized by SRSF2, which is compromised by the presence of the mutation in this region and also affects the binding of the splicing silencers hnRNP E1 and E2. The correction of both mutations was not attempted because, in both cases, the full-length transcript leads to the production of aberrant proteins that arise from a missense (c.257 C>T) or a nonsense (c.241 C>T) mutation [98]. However, the studies that were performed may still be of use to the design of ASO therapeutic strategies that involve this exon.

For the c.1122 C>T mutation, which has a silent effect on the amino acidic sequence, the possibility of redirecting the transcript processing using modified ASOs was tested in patients' fibroblasts (Figure 5). Four ASOs were used, three 2′-O-methyl (2′OMe) and one locked nucleic acid (LNA), all of which were complementary to the region of the

newly created 5′ss in order to block the access of the splicing machinery to the mutant mRNA, thus preventing the formation of the mutant transcript. Quite unexpectedly, however, this treatment failed to abolish the abnormal transcript and instead resulted in the appearance of another aberrant splicing product that corresponded to the total skipping of exon 8. Furthermore, the transfection of these ASOs in control fibroblasts also led to the appearance of the aberrant transcript that was observed in the patients' cells, which showed that oligonucleotides masked an important *cis*-acting element for the 5′ss regulation of exon 8 [98].

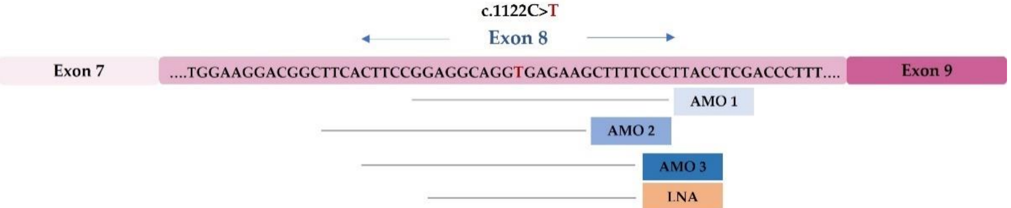


**Figure 5.** Antisense oligonucleotide (ASO) treatment for a MPS II-causing mutation: schematic representation of the *IDS* exon 8, in which the c.1122C>T nucleotide change is located (marked in red). The underlined sequences represent each blocking AMO or LNA that was designed for the different regions of the exon. Abbreviations: AMO, antisense morpholino; LNA, locked nucleic acid.

Overall, the importance of functional studies for understanding the pathogenic consequences of mis-splicing became evident from these results. Moreover, this study highlighted the difficulty in developing antisense therapies involving regions of genes that are under complex splicing regulation.

### *4.2. Development of Therapeutic Approaches Using Modified U1 snRNA Vectors: The MPS IIIC Example*

In 2014, Matos et al. showed that a modified U1 snRNA could be a promising tool for the treatment of splicing mutations in MPS IIIC patients. This was actually the first published study that assessed the potential of modified U1 snRNAs to correct of splicing mutations, not only in MPSs but also in the larger LSD field [102].

That study included five patients who carried four different mutations: c.234+1G>A, c.633+1G>A and c.1542+4dupA, which affect the donor splice site, and c.372-2A>G, which affects an acceptor splice site of the *HGSNAT* gene. For the first three mutations, different modified U1 snRNAs were designed to recognize the mutated site (Figure 6).

Again, the *in vitro* assessment was started by checking whether the splicing patterns that were observed in patients' fibroblasts could be reproduced *in vitro* in an artificial system, which would allow for the subsequent functional analysis of each target mutation. In order to reproduce the splicing defects in a cellular model, several mutant minigenes were constructed and transfected in COS-7 cells. Post-transfection cDNA analysis and sequencing disclosed that the minigene-derived splicing patterns closely resembled the patterns that were observed in the control and patients' cDNAs, which were obtained from the fibroblasts that had been previously analyzed. This observation further supported that those minigenes were reliable tools for testing and optimizing the overexpression of the modified U1 snRNAs to correct the splicing defects. So, several U1 constructs were generated with different degrees of complementarity to each mutated donor splice site. However, the splicing correction was not observed when they were tested in these artificial systems in all cases.

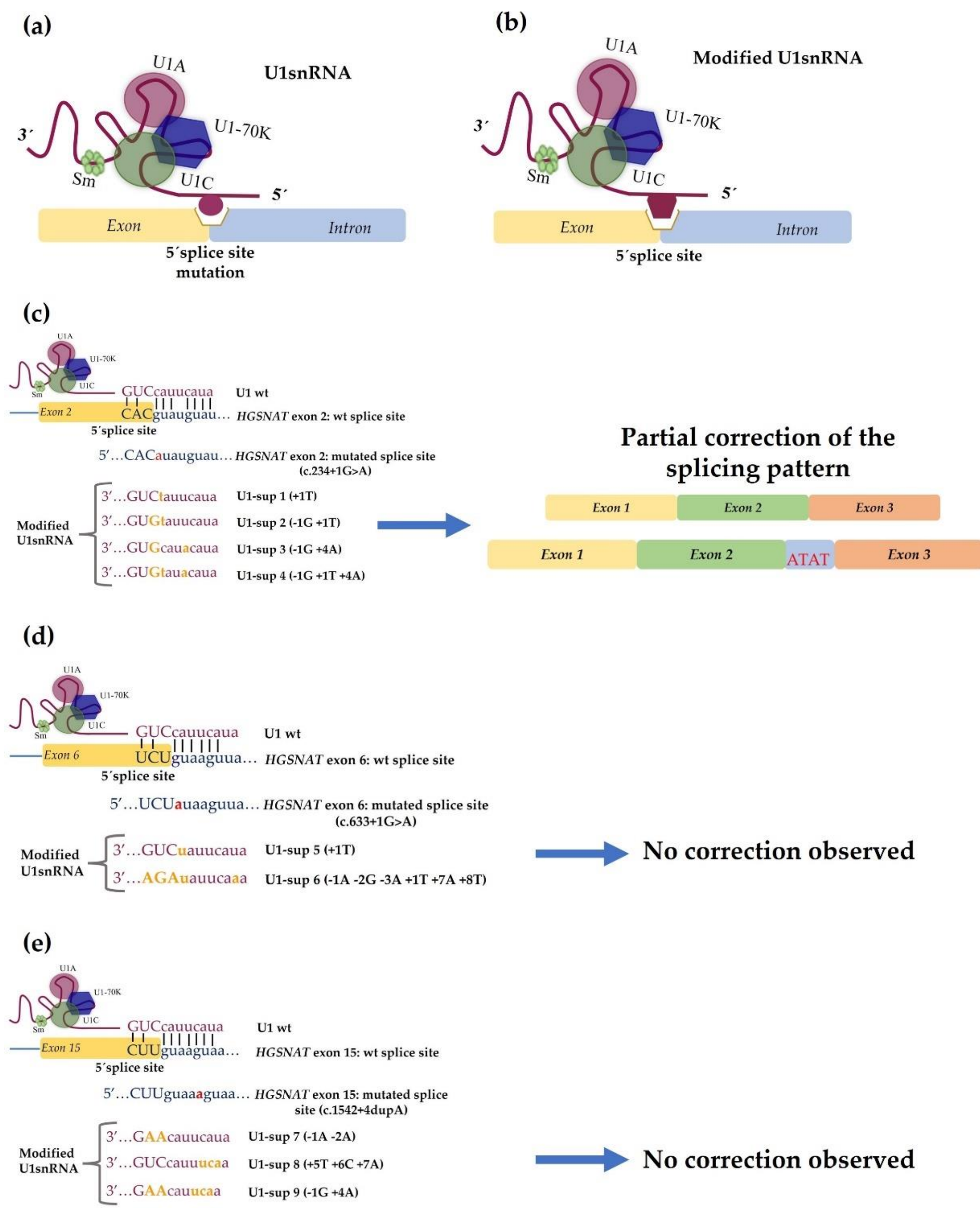


**Figure 6.** Therapeutic approach using modified U1 snRNA vectors: (**a**) the 5′ region of the U1 snRNA is involved in the recognition of the 5′ss. A mutation in this site compromises the binding of this molecule and the normal splicing process cannot occur; (**b**) a strategy for recovering the normal splicing process is the application of modified U1 snRNA to improve the recognition of the mutated 5′ss; (**c**–**e**) therapeutic approaches with different U1 snRNAs to correct the pathogenic effects of the splice site mutations in the *HGSNAT* gene (c.234+1G>A, c.633+1G>A and c.1542+4dupA). For the mutation described in (**c**), a partial recovery from the splicing defect was observed after treatment with the fully adapted U1 snRNA (U1-sup4). After sequence analysis, two different sequences were observed: one with a normal splicing pattern and another that included the first four base pairs of the intron 2 (ATAT). For the other two mutations at the 5′ss of the *HGSNAT* gene, no correction was observed after the application of the modified U1 snRNAs. Upper case letters show exonic nucleotides and lower case letters denote intronic nucleotides. Base pairing is indicated by vertical lines. The mutant nucleotide is highlighted in red and the changed nucleotides in the U1 sequence are illustrated in orange.

For the c.234+1G>A minigene, an expected band for the normal splicing was observed after co-expression with three of the five U1 snRNAs that were being tested; however, after sequence analysis, it was possible to observe that the fragment included exon 2 and the first four base pairs of intron 2 due to the use of an alternative downstream donor site (Figure 6c).

For the mutant c.633+1G>A minigene, an apparently normal band was detected with the overexpression of the U1 that matched all nucleotides of the mutated donor splice site. Yet again, the sequence analysis showed that, apart from exon 6, the first four nucleotides of the intron 6 were included. A band that corresponded to the skipping of exon 6 was also observed (Figure 6d).

In the c.1542+4dupA mutant minigene, when the co-transfection of the totally complementary U1 was performed, no correction was achieved, as the resulting fragment included not only exon 15 but also the first four nucleotides of intron 15. The inclusion of intronic nucleotides in all cases was due to the presence of a "gt" dinucleotide in positions +5 and +6 (Figure 6e).

Despite these results and taking into account that the minigenes only included partial intronic sequences that could lack some splicing regulatory sites and that they were assayed in non-human cells, modified U1 snRNAs were tested on patient-derived fibroblasts. For the c.234+1G>A mutation, a partial correction (almost 50%) was observed when the totally complementary U1 was transfected: one sequence demonstrated normal splicing and the other included the first four base pairs of intron 2 (as detected in the minigene approaches with COS-7 cells). However, no improvement in enzyme activity was observed. In the other patient fibroblasts (mutations c.633+1G>A and c.1542+4dupA), no effects of any modified U1 snRNAs were observed on the endogenous splicing process.

### *4.3. Identification and Characterization of Novel Splicing Defects and Assessment of Their Amenability for Splicing Correction Therapeutic Approaches: The MPS I Example*

While there are only two publications on the design of innovative approaches for the correction of specific splicing defects in MPSs, to the best of our knowledge, many other MPS-causing mutations could also be amenable to splicing correction therapeutic approaches, as demonstrated by the significant number of splicing defects that have been (already) identified in this group of pathologies (Table 2). Moreover, as in DMD, other mutations besides the splicing mutations could be corrected with ASOs, namely the deletions and insertions that cause frameshift and for which exon skipping approaches could be applicable. Thus, many other studies could be designed to assess the feasibility of ameliorating the phenotypes of these multisystemic diseases by "simply" either correcting, skipping or partially recovering their underlying defects. The recent developments in the broader RNA therapeutics field, together with the growing number of splicing modulation therapeutics that have either been approved or are under development, will certainly contribute to increase the number of studies using this sort of approaches and extend the catalogue of genetic diseases to which they apply.

In our lab, for example, we are also addressing another MPS-causing mutation, which is known to disrupt splicing: the c.1650+5G>A mutation in the *IDUA* gene (Figure 7). This single nucleotide change leads to exon 11 skipping and, when present in homozygosity or compound heterozygosity, causes MPS I. Being a 5′ss mutation, this pathogenic variant could be an excellent target for mutation-specific U1 snRNA-mediated therapeutic approaches. Thus, we performed this antisense snRNA therapeutic strategy on fibroblasts of a MPS I patient harboring the 5′ss mutation c.1650+5G>A in compound heterozygosity with a nonsense mutation (c.1205 G>A; p.W402X) in intron 11, which leads to the exon 11 skipping of the *IDUA* gene. Briefly, we constructed three different U1 variants with increased complementarity to the mutated 5′ss. Unfortunately, when they were transfected in the patients' fibroblasts, no correction was achieved. Instead, it was still possible to observe the skipping of exon 11 (unpublished data).

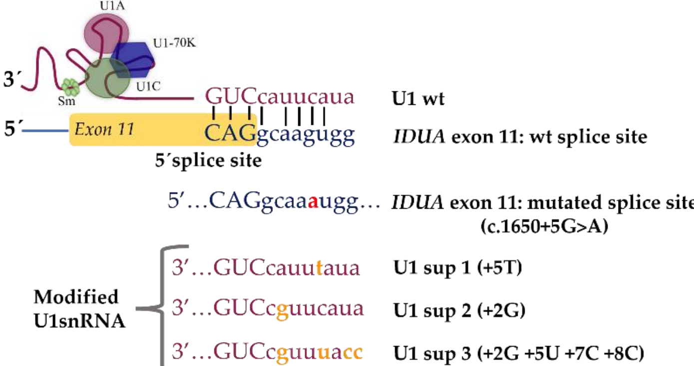


**Figure 7.** Therapeutic approach using modified U1 snRNA vectors. Different U1 snRNAs were designed to correct the pathogenic effects of the splice site mutation c.1650+5G>A in the *IDUA* gene. Upper case letters show exonic nucleotides and lower case letters denote intronic nucleotides. Base pairing is indicated by vertical lines. The mutant nucleotide is highlighted in red and the changed nucleotides in the U1 sequence are illustrated in orange.

The 5′ss is a very important sequence as it is a key factor in influencing not only the recognition of the donor splice site by U1, but also the overall success of the U1 therapeutic approach. This sequence can have a degenerative pattern feature and does not always conform to the consensus sequence (CAG/GURAG; R-purine) [103,104]. Therefore, not all positions of the sequence are equally important for enabling recognition by U1 and ensuring correct splicing. Various base pair combinations within the 5′ss can increase the U1 binding affinity [105].

Having this in mind, we are now performing further investigations. We started with one of the most obvious possibilities: the hypothesis that the absence of correction for the c.1650+5G>A mutation was caused by a low transfection efficacy. An interesting approach would be to test the therapeutic recovery of the mutation using a viral transduction technique. Viral vectors are considered significantly more efficient and less toxic than other delivery systems, namely cationic lipid transfection reagents such as Lipofectamine®. In fact, the viral transduction of U1 constructs in patients' fibroblasts has already been successfully applied for some diseases, allowing for the total or partial recovery of mis-spliced transcripts [106–108]. This is what we are currently testing in fibroblasts from MPS I patients carrying this splicing mutation. Other alternatives include testing the effects of modified U6 snRNA vectors in a similar way to that tested for the U1 snRNA vectors. Indeed, U6 snRNA has also been described as essential for proper splicing since its interaction with nucleotides at positions +4 to +6 of the splice donor site is necessary for the correct recognition of the exons at the 5′ss [109,110]. There is a published example in which only the co-application of adapted U1 and U6 isoforms corrected the splice defects that were caused by a +5 mutation [105].

Whatever the MPS we chose, the possibilities are numerous and diverse, as the catalogue of splicing defects known to cause it is vast (Table 2). Nevertheless, most of those variants are not particularly frequent among affected families. In fact, many of them are unique or rare. This could be an obstacle not only to the development of this sort of approaches, but also ultimately to making sure that those approaches that succeed eventually reach the clinic.

## 5. Challenges for the Development of Splice Modulation Approaches for MPSs

Regardless of these hurdles, MPSs, as with virtually any other LSD, are excellent candidates for splicing modulation for a number of reasons. First, they are monogenic diseases whose molecular bases have been under the lens of several teams around the world for many decades and knowledge about them has increased tremendously during this time. Second, and perhaps most importantly, it is assumed that a threshold enzyme

activity of approximately 10% is sufficient to prevent storage in LSDs [111,112]. This means that even a partial recovery could be sufficient to promote a clinically relevant effect.

Altogether, the possibilities are multiple and worth addressing. Still, there are at least two major issues that we need to address in order to ensure that this sort of therapeutic approach fulfils its full potential in the LSD field. The first, and most obvious, issue is the need for appropriate animal models in order to test these approaches *in vivo*.

### *5.1. Existence of Disease-Relevant Models*

As important as cell models may be, a significant part of the efforts to demonstrate the therapeutic potential of any drug relies on studies with model organisms. The preclinical studies of adequate animal models are a major prerequisite, not only as proof of efficacy but also for safety and toxicity assessments, which are essential for the design of subsequent clinical trials. As previously discussed, the proper *in vivo* testing of splice modulation therapeutics requires the development of animal models that carry the specific splicing mutations. In fact, even though genetic models for MPSs encompass a wide range of biological systems [113–115] thanks to the numerous advances in mutagenesis techniques that have markedly improved the efficiency of model generation, knockout or transgenic mouse models that carry null mutations remain the gold standard within the field. It is important to notice, however, that while efforts should be made to develop suitable animal models, this may not be a straightforward task given the differences in the sequences that are involved in the overall splicing processes in different species [9]. Furthermore, the numerous species-specific differences that exist in orthologue-coding sequences may also hamper the process of animal model generation.

### *5.2. Design and Development of Effective Delivery Strategies*

While the most obvious difficulty in terms of delivery is probably the BBB, which prevents patients with MPSs that involve the CNS from benefiting from several of the possible therapeutic approaches, including those which are already on the market, brain delivery may actually be feasible for some specific splicing modulation approaches. In fact, taking into account the latest findings on the wide distribution of ASOs after IT administration and its safety and tolerability, splicing modulation approaches that rely on ASOs hold a great promise for clinical translation. Nevertheless, the delivery of modified U1 snRNAs to the brain remains a pending issue. It is also important to note that brain delivery is far from being the only concern when it comes to promoting the clinical translation of this sort of approaches. There are other target tissues/organs that need to be taken into account when considering MPS-tailored approaches, namely the skeletal system. In fact, skeletal pathology is a huge burden in many MPSs and the currently available therapies fail to prevent or resolve it. The same is true for cardiovascular targeting, even though cardiovascular disease is not as prevalent in MPSs as skeletal pathology. Thus, both bone- and heart-targeting of therapeutic molecules are issues to be considered when designing splicing modulation approaches for MPS. Again, one possibility is to take advantage of the cell-specific receptors that can be targeted for uptake into these particularly impervious tissues [61].

### *5.3. Accurate Characterization of Disease-Causing Variants at mRNA Level*

Finally, there is yet another issue that should not be forgotten: our efforts to correct specific pathogenic variants should also be accompanied by a serious attempt to characterize each novel disease-causing variant more accurately. While this may sound strange in a post-genomic era in which NGS allows for multiple genes to be sequenced in parallel, assuring a faster and more efficient identification of pathogenic variants while saving time and resources, the need for in-depth molecular characterization remains an issue [116]. In fact, even though NGS technologies have contributed to greatly to enlarging the catalogue of known disease-causing variants and have actually broadened the overall number of known genetic diseases (for example, the recently identified MPS type X was actually identified

through exome sequencing,), many of those variants need to be further investigated. This is particularly relevant for the mutations that affect splicing, which have to be functionally characterized and their impact evaluated at the molecular level. In fact, DNA variants that affect mRNA expression and processing are often missed or poorly characterized, not only because they are only analyzed at the genomic level but also because certain mRNA species tend to be subjected to degradation. A recent example in the field came from our own experience in the molecular characterization of LSD patients. For example, we recently demonstrated that an *NPC1* silent variant, which was previously classified as a non-pathological polymorphism (p.V562V), actually induces exon 11 skipping, which then leads to the appearance of a premature stop codon and underlies juvenile Niemann–Pick type C disease. This work relied on a series of molecular studies and led us to revisit other Portuguese patients who had been molecularly screened for the *NPC1* gene but for whom it was not possible to establish a definitive diagnosis. By doing so, we found a second patient with a clinical presentation of Niemann–Pick type C who harbored the silent p.V562V in heterozygosity with another known disease-causing mutation [117], thus highlighting the interest of reanalyzing existing test results in known disease genes **[116]**.

Plus, a better understanding of the fine mechanisms that regulate AS will also allow for a more effective targeting of those processes, thus contributing to the design and development of novel and more effective tools for therapeutic splicing modulation.

## 6. Concluding Remarks

Several lines of evidence support the *in vivo* effectiveness of RNA-based therapies in recovering aberrant splicing and, while exploratory, the studies on MPSs tend to follow this trend. Overall, the results that were reviewed in this paper further encourage the preclinical development and testing of this sort of approaches for this group of diseases, which so far either completely lack effective therapeutic options or have an urgent need for less expensive and more effective treatment. Still, in order for these approaches to reach the clinic and fulfill their therapeutic potential, several measures need to be undertaken both before and after the *in vitro* assessments. In fact, in an era in which a single genetic analysis allows us to sequence a huge number of genes and provide fast and reliable diagnoses, DNA variants that affect mRNA expression and processing are often still missed or their effects are poorly characterized. Thus, any efforts to address the therapeutic potential of splice modulation approaches should probably start earlier, with the proper molecular analysis of disease-causing pathogenic variants, in order to better characterize the incidence of splicing mutations and better understand their impacts at the molecular level. It is also mandatory to address the subsequent need for suitable animal models and better delivery systems for *in vivo* testing.

In addition, while not discussed in this review, there is another possible way to apply splicing modulation ASOs as a potential therapeutic approach for the treatment of MPSs: to deliberately skip or promote the skipping of disease-bearing exons. This is an approach that is somehow similar to that used for the treatment of DMD patients, which we briefly summarized in our introduction section (Figure 3). This would obviously require extra caution because the removal of whole exons or series of exons may be quite deleterious. Nevertheless, it could be feasible and even advantageous in some particular cases, as long as some key requirements are met. First, it would have to be checked whether the exon skipping under consideration would give rise to an in-frame protein product because it is mandatory to keep the remaining amino acid sequence intact. Then, it would also be necessary to check which protein domains would be affected by the change and how essential they are for protein function. Skipping an exon that codes for amino acids that are directly involved in the catalytic activity core of the enzyme, for example, may have a direct impact on protein function. Therefore, a careful bioinformatic analysis should be performed before considering this approach *in vitro*. Once attempted either in patient or model cell lines, a cautious analysis of the enzyme activity, location and expression should also be undertaken. While risky, this may be yet another route to targeting MPS diseases

using splicing modulation approaches. Nevertheless, to the best of our knowledge, no-one has ever attempted this sort of therapeutic approach for MPS diseases.

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# Annex 2

**The 2020s Tooth Fairy Project:**

- Informed Consent Form
- Summary of the Project and its Objectives
- Recommendations and frequently asked questions

**CE – Comissão de Ética para a Saúde**

# DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO (1) (2)

Para participantes privados do exercício de autonomia

A Fada dos Dentes 2020

*Designação do Estudo/Projecto (em português):*

Eu, abaixo-assinado,

*(nome completo do representante do participante)*

em representação de

*(nome completo do participante privado do exercício de autonomia)*

compreendi a explicação escrita e verbal que me foi dada acerca deste estudo/projeto de investigação, tomando conhecimento dos objetivos, métodos, benefícios previsíveis e riscos potenciais, bem como das garantias de confidencialidade previstas para os dados que disponibilizo. Foi-me dado tempo de reflexão e oportunidade de fazer as perguntas que julguei necessárias, obtendo respostas satisfatórias.

Sei que tenho o direito de recusar, a qualquer momento, a minha participação no estudo/projecto através do contacto com o investigador responsável abaixo identificado, sem que isso possa ter como efeito qualquer prejuízo na assistência que me é prestada. Sei também que não serei ressarcido de quaisquer despesas decorrentes da participação.

Pretendo ser informado, através de médico referenciado, de resultados da investigação que possam vir a demonstrar-se de utilidade clínica para a sua doença – não aplicável ☐ SIM X NÃO

Se terminado este estudo/projeto ainda existir alguma das amostras biológicas facultadas, autorizo que sejam conservadas para utilização em estudos futuros, devidamente aprovados pela Comissão de Ética para a Saúde do INSA? ☐ SIM ☐ NÃO

Se SIM, pretendo que as amostras sejam tornadas anónimas de forma definitiva ☐ SIM ☐ NÃO

Autorizo a utilização dos resultados obtidos, devidamente anonimizados, para publicações científicas? ☐ SIM ☐ NÃO

Estas são as condições em que decido livremente aceitar que participe no estudo/investigação

Data: ____ / _________________ / 20____

*Assinatura do participante*

*Assinatura do investigador responsável*

**Investigador responsável**

*Nome Maria Francisca Coutinho*
*Contactos 96 786 90 01 22 340 11 00*

[1] Considerando a "Declaração de Helsínquia" da Associação Médica Mundial (Brasília 2013)
[2] Feito e assinado em duplicado sendo entregue um exemplar ao responsável, juntamente com o documento informativo.

# ELEMENTOS DA INFORMAÇÃO AO PARTICIPANTE

*A informação escrita a disponibilizar em linguagem de fácil compreensão ao participante na investigação, anexa à Declaração de Consentimento Informado e Esclarecido, deve contemplar obrigatoriamente, os seguintes pontos:*

*a. Identificação do projeto;*

**A Fada dos Dentes 2020:**

*b. Objetivo do projeto;*

O objectivo deste projecto é estabelecer **linhas celulares neuronais** a partir de células **estaminais da polpa dentária** de doentes com Mucopolissacaridose de tipo III (MPS III), ou síndrome de Sanfilippo, uma doença lisossomal de sobrecarga de apresentação neurológica, e para a qual não há, actualmente, qualquer terapia disponível.

Do mesmo modo, pretende-se também estabelecer linhas celulares neuronais de indivíduos saudáveis da mesma faixa etária (controlos), através do mesmo método.

*c. O que se pede ao participante;*

Pedimos aos participantes que estejam em fase de transição entre a dentição decídua (*dentes de leite*) e a dentição permanente (*dentes definitivos*), a **doação de um dente de leite (canino ou incisivo)** para posterior utilização como amostra biológica no âmbito deste projecto.

Importa referir que não estamos a pedir a remoção activa dos dentes. Pedimos apenas que, aquando da **queda natural de um dente de leite**, a família o recolha e preserve numa solução adequada *("solução/meio de transporte"*, que lhes será facultada aquando da assinatura da Declaração de Consentimento Informado e Esclarecido por parte do representante do participante).

Uma vez colocado o dente na *solução/meio de transporte*, pedimos aos representantes legais do participante que procedam ao envio da amostra para o nosso laboratório, sito em:
**Instituto Nacional de Saúde Dr. Ricardo Jorge**
**Centro de Saúde Pública Doutor Gonçalves Ferreira**
**Rua Alexandre Herculano, 321 | 4000-055 Porto | Portugal**

Ao cuidado de: **Maria Francisca Coutinho**
Grupo de Investigação em Doenças Lisossomais de Sobrecarga
Unidade de Investigação e Desenvolvimento
Departamento de Genética Humana

Para tal, bastará colocarem o tubo contento o dente mergulhado em *solução/meio de transporte* no envelope que lhes será entregue para esse fim aquando da assinatura da Declaração de Consentimento Informado e Esclarecido por parte do representante do participante.

Importa referir que não incorrerão em qualquer despesa, uma vez que se tratará de um envelope pré-pago com a indicação na zona de franquia “Taxa Paga”. Além disso, o envelope já estará pré-preenchido com os dados do destinatário.

*d. Benefícios esperados e riscos possíveis para o participante;*

*Benefícios directos (i.e., a curto prazo):* os participantes não terão qualquer benefício directo da sua intervenção neste estudo.

*Benefícios indirectos (i.e., a longo prazo)*: indirectamente, os participantes poderão ajudar ao desenvolvimento de abordagens terapêuticas inovadoras para MPS III e acelerar o seu processo de translação clínica, na medida em que as amostras que irão doar nos permitirão estabelecer linhas celulares mais adequadas à avaliação do impacto terapêutico a nível neuronal.

*Riscos possíveis*: não estão identificados quaisquer riscos para os participantes.

*e. Carácter voluntário da participação;*

Esta participação no projecto “*A Fada dos Dentes 2020*” é totalmente voluntária e em nada influencia a qualidade dos cuidados de saúde e acompanhamento a que o indivíduo será posteriormente sujeito.

*f. Liberdade para decidir (sim ou não) sem que se comprometa a prestação de cuidados de saúde nem o respeito pelos direitos à assistência que lhe é devida;*

Os indivíduos confrontados com o pedido de participação no projecto “A Fada dos Dentes 2020” têm o direito de recusar a sua participação no estudo/projecto, sem que isso possa ter como efeito qualquer prejuízo na assistência que lhes é prestada.

*g. Tempo disponível para refletir sobre o pedido de participação, inclusive para poder ouvir opinião de familiares e/ou amigos*

Este documento contém as informações que consideramos necessárias e essenciais à informação do(s) participante(s). No entanto, quaisquer perguntas que os participantes julguem necessário colocar, podem ser colocadas através do contacto com o investigador responsável abaixo. É também concedido, a todos os participantes, o tempo de reflexão que julguem necessário uma vez confrontados com o pedido de participação, inclusive para poder ouvir opinião de familiares e/ou amigos.

*h. Possibilidade de retirada do projeto, sem que se comprometa a prestação de cuidados de saúde nem o respeito pelos direitos à assistência que lhe é devida;*

Os participantes têm, a qualquer momento, o direito de recusar a sua participação no estudo/projecto através do contacto com o investigador responsável abaixo identificado, sem que isso possa ter como efeito qualquer prejuízo na assistência que lhes é prestada.

i. *Garantia da privacidade, confidencialidade e proteção dos dados**;

Uma vez recebidos os dentes doados por cada participante, ser-lhes-à atribuído um código alfanumérico, que nos dará indicação apenas do ano de recepção da amostra; sub-tipo da doença e número de série do caso, para cada doença.

Nenhuma outra informação será pedida e/ou mantida. Ou seja, garantiremos uma anonimização irreversível dos participantes.

* em conformidade com Lei n.º 59/2019 que aprova as regras relativas ao tratamento de dados pessoais para efeitos de prevenção, deteção, investigação ou repressão de infrações penais ou de execução de sanções penais transpondo a Diretiva (UE) 2016/680 do Parlamento Europeu e do Conselho, de 27 de abril de 2016.

*j. Informação sobre existência ou não de retribuição financeira pela participação ou de ressarcimento de despesas;*

Informam-se os participantes que não serão ressarcidos de quaisquer despesas decorrentes da participação. No entanto, importa referir que não estão previstas quaisquer despesas associadas, uma vez que a participação no projecto *"A Fada dos Dentes 2020"* não implica deslocações, ou similares.

*k. Existência de seguro (se aplicável);*

Não aplicável.

*l. Informação sobre a aprovação do projeto pela(s) CES competente(s);*

Aprovado após análise e apreciação do projeto supracitado, na reunião da Comissão de Ética para a Saúde (CES) no passado dia 23/6/2020, envia-se abaixo o parecer emitido por esta Comissão:

*"Considerando a natureza do material biológico e dados a utilizar, as circunstâncias da sua obtenção e os objetivos e métodos do estudo, a CES_INSA é de parecer que a sua realização não levanta objeções de natureza ética."*

m. Identificação do investigador responsável e forma de ser contactado;

Investigador Responsável:
Maria Francisca Coutinho
Grupo de Investigação em Doenças Lisossomais de Sobrecarga
Unidade de Investigação e Desenvolvimento
Departamento de Genética Humana

Contactos:
TEL +351 223 401 100
MÒVEL +351 967869001

*n. Modo de comunicação aos participantes e publicação dos resultados do projeto.*

Em termos científicos, os resultados do projecto serão publicados em revista internacional (língua inglesa) com revisão por pares, preferencialmente em regime *'open access'*, de modo a atingir um público o mais vasto possível.

A nível institucional, os resultados serão também divulgados através do site do INSA I.P., preferencialmente sob a forma de publicação de um artigo em língua Portuguesa, no Boletim Epidemiológico *Observações*.
Mais se informa, relativamente à publicação de resultados, que:

- Em nenhum local da publicação estará o nome de nenhum dos participantes, procurando-se por todos os meios garantir anonimidade.
- O texto será devidamente editado para se adequar à publicação selecionada tendo em conta estilo, construção gramatical, extensão, etc.
- A informação publicada, em papel ou por acesso na internet, pode ser acedida em qualquer parte do mundo e ainda que seja principalmente dirigida a médicos e investigadores pode ser lida por muitos não médicos, por exemplo, jornalistas.
- A informação, no todo ou em parte, poderá ser divulgada noutras publicações do grupo editor, de acordo com as regras de licenças existentes mas está excluída a sua utilização em publicidade ou fora do contexto.
- Pela publicação dos dados, os participantes não receberão qualquer compensação financeira ou de outra natureza.
- Mais uma vez, o consentimento dos participantes pode ser revogado até que a informação esteja em publicação, momento a partir do qual não poderá já ser impedida a sua divulgação.

## Quem somos:

Somos um grupo de Investigação & Desenvolvimento em **Doenças Lisossomais de Sobrecarga (DLS)**, sediado no Instituto Nacional de Saúde Doutor Ricardo Jorge, no Porto.

Liderado pela Doutora Sandra Alves*, o nosso grupo tem-se dedicado há mais de uma década ao estudo de uma série de DLS. As Mucopolissacaridoses, no entanto, têm sido um dos alvos preferenciais do nosso estudo.

No início da década passada dedicámo-nos a estudar as bases moleculares da doença, estabelecendo diagnósticos e analisando amostras de doentes Portugueses, de modo a perceber, ao nível do DNA/RNA qual o defeito genético de cada um. Mais tarde, decidimos usar esse conhecimento para tentar desenhar abordagens terapêuticas (também elas de base molecular) para tentar ultrapassar os defeitos moleculares que tínhamos identificado.

É no seguimento desses estudos, de resultados muito promissores, que surge este projecto, para o qual estamos a recrutar colaboradores.

Por isso, estamos a pedir a colaboração de famílias com crianças com MPS que nos queiram ceder um dentinho.

Se está interessado(a) em colaborar connosco, não hesite em contactar-nos!


## Contactos:

Maria Francisca Coutinho (francisca.coutinho@insa.min-saude.pt)

Sandra Alves* (sandra.alves@insa.min-saude.pt)



## Agradecimentos

*Fontes de Financiamento:*

*Apoio:*

## *Pedido de Voluntários:*

Projecto

# A Fada dos Dentes 2020:

*um método para estabelecer* ***linhas celuleres neuronais***

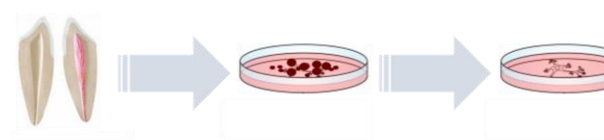
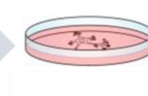
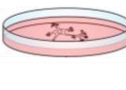
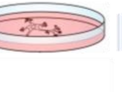
*a partir de dentes de leite de doentes com Mucopolissacaridoses*


*[1] Unidade de Investigação e Desenvolvimento, Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge, Porto, Portugal*

*[2] Centro de Estudos de Ciência Animal , CECA-ICETA, Universidade do Porto, Porto, Portugal*


Este é um projecto **inovador**, cujo objectivo é utilizar dentes de leite (caninos ou incisivos) de doentes com Mucopolissacaridoses (MPS) para gerar neurónios.

Esses neurónios serão posteriormente utilizados como modelo celular para testar abordagens terapêuticas inovadoras e tentar compreender o seu impacto ao nível do cérebro – um dos órgãos afectados nestes doentes e, para o qual ainda não há tratamento.

Para participar, basta ceder-nos um canino ou incisivo, logo que ele caia!

(ver detalhes na página seguinte)

## Um projecto

*Instituto Nacional de Saúde Doutor Ricardo Jorge*

# Perguntas frequentes (FAQs)

*Qual é o problema que estamos a tentar resolver?*

A necessidade de **modelos celulares** adequados para testar o potencial de uma série de **drogas inovadoras**, cujo objectivo é actuar ao nível dos **sintomas neuronais**, presentes em muitas MPS.

É verdade que temos resultados promissores em células de pele de doentes (fibroblastos) mas o ideal será testar as drogas directamente nos seus tecidos/órgãos-alvo. Logo, nada melhor do que testá-las directamente em **neurónios**!

*Qual é a vantagem de usar dentes de leite?*

É uma amostra **não invasiva** e totalmente **isenta de dor** para o doente!

Nada de biópsias dolorosas! Os dentinhos caem de forma natural. Tudo o que a família tem de fazer é recolhê-los, colocar num líquido próprio que vamos preparar (chamado *meio de transporte*), e enviá-los para o nosso laboratório.

*É difícil recolher/guardar os dentes?*
*Precisam de ter algum cuidado especial?*

Nem por isso mas verdade é que, para conseguirmos fazer o nosso trabalho no laboratório, há duas condições muit(íssim)o importantes:

- ✓ o dente deve ser colocado no *meio de transporte* no **máximo 20 min** depois de ter caído!
- ✓ tem de ser mandado para o laboratório nas **primeiras 48h**!

*Quanto tempo demora até conseguirmos gerar neurónios a partir de dentes?*

Embora tenhamos a ajuda de uma '*fada dos dentes*' muito especial, a verdade é que, no laboratório, nada surge '*a toque de varinha mágica*'; tudo leva o seu tempo.

No entanto, podemos dizer que, em condições ideais, o protocolo completo desde a chegada do dente ao laboratório até à obtenção de uma linha neuronal em cultura, leva no mínimo 6 a 8 semanas.

*Mas…como é que a conseguimos gerar neurónios a partir de dentes, afinal?!*

Claro que o processo laboratorial não é fácil de explicar, mas podemos dizer a coisa mais importante: os dentes de leite têm, no seu interior, uma categoria especial de células, chamadas '**células da polpa dentária**'. Algumas dessas células têm uma caraterística muito especial: são **células estaminais**.

A propriedade chave de todas as células estaminais é serem *indiferenciadas*. Isto significa que se conseguem replicar indefinidamente e substituir/renovar muitos tipos de células danificadas no organismo. Além disso, se soubermos dar-lhe o tratamento adequado no laboratório, conseguimos levá-las a dar origem a células especializadas, como é o caso dos **neurónios**!

Será garantido o estrito cumprimento de todos os requisitos legais, nomeadamente os relativos à proteção de dados, tais como a Diretiva comunitária de proteção de dados (Diretiva 95/46/EC do Parlamento Europeu e do Conselho de 24 de Outubro de 1995 sobre a proteção de indivíduos no que refere ao processamento de dados pessoais e à livre utilização dessa mesma informação) e as suas transposições nacionais. Neste contexto, será requerida uma autorização à Comissão Nacional de Proteção de Dados (CNPD) para recolha e processamento de dados pessoais, sempre que aplicável.

# INSTRUÇÕES

## ANTES de o dente cair:

- Guardar o frasco contendo o meio de transporte no **frigorífico**.
- Esperar

## DEPOIS de o dente cair:

- Passar o dente por **água corrente** (*opcional*).
- Tirar o plástico que envolve a rolha do frasco e colocar o dente lá dentro, imerso no líquido.
- Usar o filme de parafina plástica que está dentro do saco onde enviámos o frasco para selar novamente a rolha.

  *Como?*

  Destacar o papel (branco) do plástico (transparente). Uma vez separados, usar os dedos para esticar um pouco o plástico até ele se tornar maleável e contornar a rolha com ele, fazendo sempre pressão para que o plástico adira ao frasco.

- Enviar para o laboratório, usando o envelope tamanho A5 que enviamos juntamente com o **líquido de transporte**.

## Muito importante!

- O dente deve ser colocado no meio de transporte no máximo 20 min depois de ter caído.
- Tem de ser mandado para o laboratório nas primeiras 48h, juntamente com o Consentimento informado assinado.

# Annex 3

Recipes for Dental Pulp Stem Cell (DPSCs) Lines:

- Transport Medium
- DPSCs Culture Medium
- DPSCs wash Medium
- Freezing Medium

Annex 3- Recipes for Dental Pulp Stem Cells (DPSCs) Lines:

3.1. *Transport medium (stored at ≈ 4ºC):*

- 500 µL Antibiotic (PenStrep) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- 500 µL Fungizone (Amphotericin B) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- Saline solution to a total volume of 100mL.

3.2. *DPSCs culture medium (stored at ≈ 4ºC):*

- 500 µL Antibiotic (PenStrep) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- 500 µL Fungizone (Amphotericin B) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- 10 mL Fetal Bovine Serum (FBS) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- *Dulbecco's Modified Eagle Medium* (DMEM/F12 (1:1); [+]Glutamax [+] 2.438 g/L Sodium Bicarbonate) (Gibco® Life Technologies, Carlsbad, California, United States of America) to a total volume of 100 mL.

3.3. *DPSCs wash medium (stored at ≈ 4ºC):*

- 500 µL Antibiotic (PenStrep) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- 500 µL Fungizone (Amphotericin B) (Gibco® Life Technologies, Carlsbad, California, United States of America);
- Saline solution to a total volume of 100mL.

3.4. *Freezing medium (freshly prepared):*

- 150 µL DMSO;
- 550 µL DPSC culture medium.