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**Neonatal transplantation of umbilical cord blood
as a new therapeutic option
for Mucopolysaccharidosis type I**

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CHAPTER 1

General introduction

1. HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) consists in the infusion of hematopoietic stem cells (HSCs) in a patient, with the aim of re-establishing the hematopoietic function. This therapy can be employed to treat malignant pathologies, such as leukemia (acute lymphatic leukemia, acute or chronic myeloid leukemia) or lymphoproliferative disorders (Hodgkin or non-Hodgkin lymphoma, multiple myeloma, myelodysplastic syndromes, acute myelofibrosis), or to correct non-malignant disorders, such as metabolic diseases, primary immune deficiencies and hemoglobinopathies^{1,2}.

Patients that undergo HSCT are first submitted to a conditioning procedure, to totally or partially ablate their hematopoiesis. This allows the substitution of recipient's HSCs with donor ones, and guarantees sufficient immunosuppression to prevent transplant rejection.

In the case of malignant diseases, patients to be transplanted are pre-conditioned by a procedure aimed at fully eliminating the affected bone marrow. These so called myeloablative regimens are designed to kill all recipient's BM cells. They can be performed by high-dose whole body radiations, and/or by administering various combinations of chemotherapeutic agents such as busulfan, cyclophosphamide and others. In these circumstances, the purpose of the treatment is to substitute completely recipient's bone marrow with healthy donor cells, in order to avoid any relapse.

In non-malignant pathologies, instead, the aim of the transplantation procedure is different: patients receive a milder conditioning procedure than the one performed for malignant diseases, because the aim of the

therapy is to remove genetically abnormal HSCs to make room for healthy donor cells able to produce the defective protein. The non-myeloablative regimens employed in non-malignant patients are only immune-suppressive, not myelo-ablative. They are obtained administering lower doses of chemotherapeutic agents or radiations than the ones employed for myelo-ablative conditioning³.

Regarding the origin of cells to transplant, HSCT can be autologous, which means that the patient is transplanted with his/her own stem cells, or heterologous (allogenic), when the stem cells come from a donor that is not the patient.

One of the most important factors that must be considered in the choice of a donor is the degree of HLA (Human Leukocyte Antigen) match between donor and recipient. In autologous HSCT there is no possibility of HLA mismatch, because the same person acts as donor and recipient. In the case of heterologous transplant, instead, the HLA match between the two individuals must be high, to diminish the risk of rejection and graft versus host disease (GVHD), that are two main issues of HSCT-based therapies.

In the context of non-malignant pathologies, allogenic HSCT is usually performed, to substitute host genetically defective cells with cells coming from a healthy donor. Transplantation from sibling individuals is frequently considered, because they often have a sufficient degree of HLA match with the recipient. However, the outcome can be non-optimal if the donor is a healthy carrier of the disease (and so his/her enzymatic activity is only 50% with respect to non-carrier healthy donors). An emergent and promising possibility is the genetic correction of patient's own stem cells, that allows the treatment of these

diseases with an autologous transplantation (see paragraph 3.3 section “*Ex vivo* gene therapy”).

The sources of HSCs currently employed in clinic are bone marrow (BM), mobilized peripheral blood, and umbilical cord blood (UCB)⁴. BM is the traditional source of HSCs for transplantation, because in post-natal life HSCs settle in the BM. BM-HSCT is also called bone marrow transplantation (BMT). UCB, instead, is the most recently discovered source, and the transplantation from this source is called umbilical cord blood transplantation (UCBT).

Until 1988, BM was the only source employed for transplantation, even if it is often hard to find BM donors compatible with the recipients. UCB was considered a waste product, because its biological properties were not known and characterized. However, when the first UCBTs gave good results, UCB started to be employed as an alternative to BM⁵.

1.1 Umbilical cord blood as a source for HSCT

Umbilical cord blood represents a valid source of HSCs for patients affected by diseases treatable by transplantation, especially pediatric ones. Even if HSCs are normally located in the BM in post-natal life, they can be found immediately after birth in the fetal blood that flows in the umbilical cord vessels, while they are completing their migration from the liver to the BM. Yet, HSCs homing in fetal BM is a dynamic process: especially in late prenatal life, some HSCs are still moving in and out the BM, and their presence in bloodstream is much higher than in postnatal life. For this reason, a good amount of them can be

harvested perinatally, collecting the precious blood flowing in the umbilical cord.

Regarding their morphology, UCB-derived HSCs are small cells with little cytoplasm, in which mitochondria and endoplasmic reticulum are not easily distinguishable. At the nuclear level, UCB-HSCs are characterized by long telomeres that give them a more lasting hematopoietic capacity with respect to HSCs from other sources. Like all HSCs, UCB-HSCs are in the G0 phase of the cell cycle, and they have very low metabolic activity, but they are able to proliferate, to self-renew and to differentiate in cells belonging to the various hematopoietic lineages. The absolute number of HSCs in a UCB unit is quite low with respect to other sources such as BM, but this does not limit the engraftment ability into the recipients^{6,7}.

Concerning the immuno-phenotype of HSCs and other hematopoietic progenitors, the evaluation of CD34 and CD38 antigens is significant even in UCB⁸. Human HSCs purification, in fact, requires the expression of different cell surface markers, among which the most important is CD34. It is a transmembrane glycoprotein belonging to the class of sialomucine adhesion molecules, and it is involved in the regulation of cell adhesion to the stroma⁹. For transplantation aims, CD34 is the main marker to consider, together with CD38, since only CD34⁺CD38⁻ cells have long-term self-renewal and engrafting ability^{7,10}. To test the proliferative potential of CD34⁺CD38⁻ HSCs, the colony-forming cell-assay (CFC-assay) is frequently employed. The analysis of the CFCs that originate from in 1 mL of human UCB shows that this source is enriched in high proliferative potential-colony

forming cells (HPP-CFCs), that maintain stem properties and do not differentiate towards any hematopoietic lineage^{8,11}.

The first cord blood transplantation was executed in Paris in 1988, in a 5-year-old patient with a severe form of aplastic anemia, Fanconi anemia¹². UCB was collected when the recipient's sister was born, and was cryopreserved. The patient was conditioned by low-dose cyclophosphamide (20 mg/kg) and 5 Gy irradiation. At day +22 after intravenous cell infusion, the first engraftment evidence was observed, followed by complete hematopoietic repopulation and donor chimerism. The patient did not develop GVHD, and he is actually healthy, showing a complete hematopoietic and immunological long-term engraftment^{5,13,14}.

Among the most representative retrospective studies on UCBT, Rocha *et al.* in 2001 published a work, in which they compared the outcome of UCBT or BMT in pediatric patients with acute leukemia. Patients who underwent UCBT had delayed neutrophils and platelets reconstitution, lower risk of GVHD, and lower rate of relapse, compared to BM recipients¹⁵. Michel *et al.* demonstrated in 2003 that UCBT from allogeneic donors in young patients with acute myeloid leukemia was associated with a lower rate of GVHD and did not led to higher relapse risk with respect to BMT¹⁶.

1.2 Umbilical cord blood and bone marrow: comparison of the two sources

UCB is surely the most attractive source of cells for HSCT alternative to BM, especially for pediatric patients, because it shows various advantages. The main benefits of UCB compared to BM as a source of HSCs are^{5,17-19}

- UCB is easy to obtain, without any risk or harm to the donor, and without ethic issues.
- UCBT shows a lower rate of acute and chronic GVHD, due to the immaturity of its T lymphocytes, that have a lower allo-reactivity compared to the ones of peripheral blood or BM.
- The tolerable HLA mismatch between donor and recipient is higher, and the probability to find compatible donors augments, even in the case of transplantation for ethnical minorities.
- The probability of transmitting somatic mutations or infections (Cytomegalovirus in particular) is lower, because it is unlikely that these events had already happened during fetal life.
- The cryopreservation and the recovery of UCB-HSCs is an efficient process.
- The availability of UCB units is more rapid, thanks to the less stringent donor choice and to the existence of UCB banks full of stored units. This allows ready HSCT intervention, which is a big advantage, especially in the case of transplantation for metabolic diseases, in which early treatments guarantee better results.

- UCB engraftment ability overcomes the one of BM, and the probability to obtain a full donor chimerism is higher.
- In metabolic disorders, it is more frequent to obtain normal enzyme levels following UCBT than after BMT.

UCBT has, indeed, also some disadvantages^{4,5,20,21}:

- UCB gives a delayed immunologic reconstitution, with later granulocytes, neutrophils, and platelets engraftment, and longer aplasia duration.
- The total cell number of a UCB unit is lower than the one of BM.
- Only one donation per donor is possible.
- The efficacy of the adaptive immunity is lower, which could lead to viral reactivations.

1.3 Umbilical cord blood transplantation (UCBT) in non-malignant diseases

Umbilical cord blood transplantation is actually applied to various non-malignant diseases, such as haemoglobinopathies (thalassemia major, sickle cells anemia, congenic neutropenia), immune deficiencies (bone marrow aplasia, congenic immune deficiencies, Wiskott-Aldrich syndrome, auto-immune disorders) and metabolic disorders (osteopetrosis, mucopolysaccharidoses, leukodystrophies, and others)^{1,2}.

The conditioning regimen for these diseases is immune suppressive, but not myelo-ablative, because the main aim of UCBT is the correction of the genetic defect, by the eradication of genetically abnormal cells, to

make room for donor healthy ones³. The correction happens both in a direct way, by the repopulation of the recipient's hematopoietic and immune system, and in an indirect way, by delivery of the deficient enzyme to host cells through their plasma membrane²².

Generally, an allogeneic UCB donor is chosen, who has a HLA match of at least 4/6 *loci* with the recipient, and the UCB unit must contain before cryopreservation a number of nucleated cells (NC) that guarantees a dose of 3×10^7 NC/kg at least^{22,23}.

UCBT is particularly important in the treatment of inherited metabolic disorders (IEMs), a wide group of genetic diseases due to defects in genes that encode for enzymes deputed to convert some molecules (substrates) into others (products). In the majority of metabolic disorders, a genetic defect in an enzyme leads to the accumulation of its substrates, which is harmful for cells and tissues, or to a reduction in the synthesis of essential molecules²⁴. HSCT is the only clinical treatment able to guarantee long-term metabolic correction and amelioration of neurocognitive and functional problems. Following transplantation, donor cells induce the “cross-correction” phenomenon, by which the proximity of healthy cells to abnormal ones can correct the biochemical consequences of their enzymatic deficiency (see paragraph 3)^{25,26}. Moreover, hematopoietic cells can become part of non-hematological organs, such as microglia in the brain, alveolar macrophages in the lungs, and Kupffer cells in the liver²⁷.

An early diagnosis of these disorders is of main importance, because it can allow a ready intervention and the prevention of worsening damages. UCBT is actually preferable with respect to other allogeneic HSCs

sources, especially in the case of pediatric patients, for the reasons that were mentioned in the previous paragraph^{19,22}.

1.4 Murine models of UCBT

Many strategies are under development in order to improve actual UCBT approaches, and convenient animal models, primarily murine, are essential to investigate these developing strategies, to facilitate the testing and the defining of optimal treatment conditions^{5,18,21}. Indeed, the process of hematopoietic development is comparable in all mammals. It consists in a series of spatially and temporally regulated waves of generation of hematopoietic precursors, resulting, in a later stage, in the colonization of fetal liver, and finally in the migration of HSCs into the BM, where they will settle and remain during postnatal life²⁸⁻³⁰.

In mouse embryo, the hematopoietic process happens in an asynchronous way in different sites (*Figure 1*). Hematopoiesis starts at embryonic day E7.5, in the blood islands of the vitelline sac, with the generation of primitive erythroid progenitors^{31,32}. These cells support embryonic growth by oxygen supply. They are thought to derive from mesodermal precursors, not from some HSCs, because HSCs do not seem to be present at this developmental stage^{33,34}. Soon after the formation of primary erythrocytes, multipotent and lineage-restricted hematopoietic progenitors appear in different embryonic sites: the yolk sac, the peri-aortic splanchnopleura (PAS) or aorta-gonad-mesonephros region (AGM), the umbilical arteries and the placenta³⁵. These progenitors are transient and they give rise to a “primitive”

hematopoiesis. The yolk sac contains the majority of the hematopoietic progenitors at day E10, and the hematopoietic stem and progenitor cells (HSPCs), that expand and differentiate here. These progenitors are different from the primitive erythro-myeloid cells that circulate in the embryo³⁶. At day E14.5, HSPCs localize in the liver. Soon after liver colonization, the most differentiated progenitors colonize also the thymus and the spleen³⁷. In an unsettled moment a few days before birth, which usually happens at day E21, HSPCs start to migrate from the liver to the BM, where they will settle and differentiate into the various hematopoietic lineages. Primary hematopoiesis is replaced by the “definitive” one, and definitive HSCs appear³⁸. At this time, liver progenitors become quiescent: the BM is the only organ where blood cells will be synthesized during adult life³⁹. In this phase, indeed, it is possible to collect HSCs from UCB, because they circulate in the bloodstream in their migration from the liver to the BM⁴⁰.

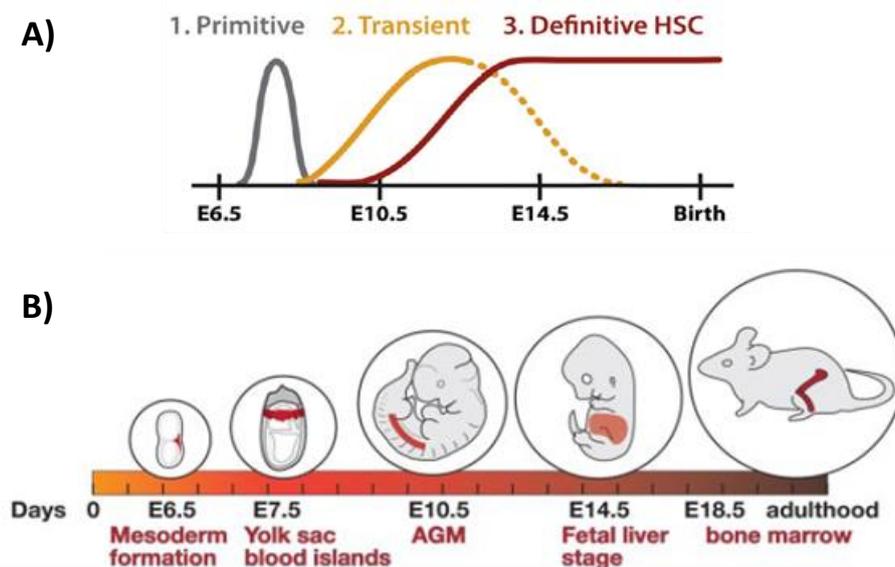


Figure 1. Murine hematopoietic development. **A)** During development, hematopoietic cells are generated in three phases that partially overlap. The first phase is transient, it starts at day E7 in the blood islands of the vitelline sac. The second is again transient, and it is defined “primitive hematopoiesis”, it starts at day E8.5 and it produces HSPCs similar to the adult ones. The third phase, the “definitive hematopoiesis”, generates definitive HSCs, beginning from E10.5⁴¹. **B)** Another representative scheme of fetal murine hematopoiesis: mesoderm formation (E6.5), blood islands development in the yolk sac (E7.5), HSCs appearance in the AGM region (E10.5), fetal liver hematopoiesis (E14.5), and hematopoiesis in the BM during late gestation (E18.5) and in the adult animal²⁸.

As shown by Migishima *et al.* in 2003 by cytofluorimetric techniques, nucleated cells purified from murine UCB at day E18.5 belong to various hematopoietic populations⁴⁰. Lymphoid T and B cells are few, but detectable, while myeloid cells (macrophages and granulocytes) are present at higher percentages. Immune cells, indeed, could be unnecessary in this phase, thanks to maternal protection. Immature erythrocytes represent, instead, the most abundant population (over 80%). The authors also tried to evaluate the presence of HSPCs with the canonical LSK phenotype (Lineage⁻, Sca-1⁺, c-Kit⁺) described for adult HSPCs, but they observed such low numbers of these cells, compared with adult BM samples, that they hypothesized HSCs at this developmental stage have a different and still unknown phenotype.

In the literature, very few papers are present that concern murine UCB and its transplantation. More often, in fact, studies are carried on human UCB that, however, can only be transplanted in immune deficient mice. However, immunocompromised mouse models are not available for every disease, so the development of a murine-to-murine UCB transplantation is an interesting and useful matter. Murine UCB

contains very low cell numbers, so some studies about fetal murine HSCs have been performed employing other comparable sources, such as fetal liver cells (FLCs) at day E14-E16. However, FLCs are susceptible to a higher reject risk, and, most importantly, they are not a completely representative model of the UCB collected at birth, since they come from a solid organ and they are harvested in a more precocious developmental phase^{42,43}.

2. MPS-I DISEASE

2.1 Lysosomal diseases

Lysosomal diseases (LDs) are a subgroup of inborn errors of metabolism (IEMs) characterized by defects in specific lysosomal hydrolases and resulting in intracellular accumulation of unmetabolized substrates. Most LDs result from mutations in genes that encode for soluble lysosomal acid hydrolases, while a few result from defects in genes encoding for membrane proteins essential to the function of the lysosomal system⁴⁴.

These disorders are individually rare, but their combined frequency is almost 1 in every 8000 live births. They are usually inherited in an autosomal recessive fashion, except for Fabry, Hunter and Danon diseases, which are X-linked⁴⁴.

The classification of LDs is mainly based on the nature of the primary storage material that accumulates within the lysosomal compartment as a result of the genetic defect.

Lysosomes are the main degradative organelles in all cells. The catabolic process that occurs within them is performed by soluble enzymes contained in this organelles and called lysosomal acid hydrolases. These enzymes are synthesized on the surface of the rough endoplasmic reticulum (RER), and then they move to the *cis* Golgi network, where they are added a phosphate group on some mannose residues. The resulting mannose-6-phosphate (M6P) groups are bound by M6P receptors (MPRs) and gathered into clathrin-coated vesicles, that bud from the Golgi and fuse with late endosomes. Some hydrolases,

although M6P-tagged, escape binding to MPRs and are directed by default to the cell surface and secreted. Anyway, since some MPRs are localized at the plasma membrane, the enzymes can be re-uptaken and they can reach the lysosomes by receptor-mediated endocytosis^{45,46}.

The degradation of substrates that occurs in the lysosomes is a stepwise activity that requires the sequential action of a series of lysosomal hydrolases: if a step in the process fails, further degradation is impossible and partially degraded substrates accumulate. For this reason, mutations in genes that encode for lysosomal hydrolases or proteins that are necessary for their post-translational modification or transport often result in LDs⁴⁷.

The main explanation of the pathogenesis of LDs is that the primary enzyme deficiency causes the intra-lysosomal accumulation of the substrates normally degraded by the lacking enzyme, leading to lysosomal engulfment. This affects cell architecture and function, leading to cell death. In the last few years, other numerous and interconnected cellular pathways, perturbed in LDs as a result of the primary lysosomal defect, are being identified. The most notable and studied are autophagy block, inflammatory and apoptotic response, biochemical injury due to toxic metabolites accumulation, derangements in pH regulation, calcium and iron homeostasis, abnormalities in endoplasmic reticulum stress responses, and energy failure⁴⁸⁻⁵².

The resultant clinical phenotype reflects the amount of residual enzyme activity and the pattern of cell types involved. As lysosomes are ubiquitous organelles, which role is crucial in almost all cell types, however, LDs are typically multi-system diseases. Even if LDs are

heterogeneous in terms of age of onset, clinical features and rate of disease progression, most of them are characterized by pediatric onset, progressive course, and significant morbidity, with abnormalities of the nervous system, viscera, muscle, bone and cartilage, and reduced lifespan^{44,53,54}.

2.2 Mucopolysaccharidoses

Mucopolysaccharidoses (MPSs) are a prominent subgroup of lysosomal diseases. They result from the deficient activity of enzymes required for the catabolism of glycosaminoglycans (GAGs, once known as mucopolysaccharides) that are long unbranched polysaccharides, ground substance of all connective tissues. The degradation of GAGs requires eleven enzymes⁵⁵. The deficiency of each of them has been associated with a specific MPS disease, so, to date, eleven MPSs are recognized: MPS type I, II, III (with four subtypes, A, B, C and D, each caused by a defect in a different enzymes), IV (with two subtypes, A and B), VI, VII, and IX. All MPSs show an autosomal recessive pattern of inheritance, except for MPS type II, which is X-linked. MPSs are rare diseases: the overall incidence of MPSs as a group is estimated to be approximately 1 in 25000 live births⁵⁵.

Glycosaminoglycans (GAGs) are organic macromolecules consisting of long unbranched polysaccharides, constituted by many disaccharide units. One component of the disaccharide unit is always an amino sugar, from which the name “glycosaminoglycans” comes. GAGs often exhibit a high rate of sulphation, which gives them a negative electric charge and renders them highly water-attractive⁵⁶. The majority of

GAGs are covalently linked to core proteins, forming large complexes known as proteoglycans. Alone or as proteoglycan components, GAGs are the main constituents of the extracellular matrix of all connective tissues. They are abundant in skin, cartilage, bone, tendons, blood vessels, and in the stroma of all organs. Their mechanical role depends on their capacity to retain water and on their elastic properties: they form a jelly-structure with exceptional lubrication and shock-absorbing capacity, guaranteeing also the maintenance of a hydrated organized extracellular space, in which nutrients and signalling molecules can move. Therefore, they fulfil many physiological functions, modulating processes such as cell adhesion, motility and response to damage, growth factors and cytokines signalling, bone formation and remodelling^{55,57}.

GAGs are continuously renewed, part in the lysosomes (following their uptake by endocytosis), and part extracellularly (by secreted enzymes). In MPSs, the lack of GAGs degradation due to lysosomal enzyme deficiency leads to GAGs storage, which results in cell, tissue and organ dysfunction, determining the clinical symptoms observed in patients, with increased GAGs accumulation in the extracellular spaces and GAGs urine excretion⁴⁵. MPSs are usually characterized by a chronic and progressive course, with different age of onset and velocity of progression depending on the severity of the defect. Since GAGs role is prominent in cartilaginous tissue, MPS patients typically develop a series of bone and joint abnormalities, but GAGs accumulation affects all connective tissues, so MPSs have the characteristics of multi-system diseases. The most common manifestations are short stature, coarsened facial features, mental retardation, deafness, corneal clouding, bone and

joint features, respiratory infections, abdominal protuberance due to liver and spleen enlargement, recurrent inguinal or umbilical hernia⁵⁷.

2.3 Mucopolysaccharidosis type I (MPS-I)

Mucopolysaccharidosis type I (MPS-I) is one of the most frequent lysosomal disorders. It belongs to the group of Mucopolysaccharidoses, and it is inherited in an autosomal recessive fashion. MPS-I patients present mutations in the gene encoding for the enzyme alpha-L-iduronidase (IDUA), that is involved in the degradation of the GAGs heparan sulphate (HS) and dermatan sulphate (DS). A low or absent IDUA activity leads to the accumulation of these GAGs in the lysosomal compartment, in the extracellular spaces and in biological fluids⁵⁸.

This disease was first described in 1919 by the German pediatrician Gertrude Hurler, who reported the case of two unrelated boys affected by visceromegaly and bone abnormalities, and was named “Hurler syndrome” after her⁵⁹. It was also referred to as “Gargoylism”, because of the coarse facial features of affected children, that reminded the grotesque figures of the gothic cathedrals⁶⁰. In 1962, Harold Scheie described an attenuated phenotype of Hurler disease, that was thus called “Scheie syndrome”⁶¹. A few years later, Wiesmann and Neufeld showed that Hurler syndrome and Scheie syndrome resulted from a deficiency of the same enzyme⁶². In 1972, Hurler syndrome, Scheie syndrome, and a series of other phenotypes of intermediate gravity between the two, were all classified as MPS type I, with three recognized clinical subtypes: MPS-I-H (Hurler syndrome, OMIM

#607014, the most severe form), MPS-I-H/S (Hurler/Scheie syndrome, the intermediate form), and MPS-I-S (Scheie syndrome, the most attenuated form)⁶³.

MPS-I is the MPS with the largest number of diagnoses worldwide. The overall prevalence of this disease is 1:100000 live births. MPS I-H is the most common subtype, with a prevalence of 7.6:10000⁶⁴.

2.4 Molecular bases and phenotypic determination of MPS-I

IDUA enzyme (EC #3.2.1.76) is an ubiquitous lysosomal acid hydrolase which catalyzes the hydrolysis of unsulphated alpha-L-iduronosidic linkages, removing the terminal alpha-L-iduronic acid residues from the GAGs DS and HS⁶². It was originally purified from human urine in 1971 by Barton and Neufeld, who defined it “Hurler corrective factor”, because, when it was added to cultured Hurler fibroblasts, they acquired the ability to degrade sulphate mucopolysaccharides⁶⁵. IDUA is a monomeric soluble protein with a molecular mass of 82 kDa, and its primary structure consists of 653 aminoacids⁶⁶.

The human *IDUA* gene maps to chromosome 4p16.3, from base pair 980784 to base pair 998316⁶⁷. In 1992, Scott demonstrated that the length of this gene is about 19 kb and that it is made up of 14 exons⁶⁸. To date, the Human Genome Mutation Database accounts for 249 possible mutations in *IDUA* gene, the majority of which are nonsense mutations, missense mutations, small insertions or deletions, and splice site mutations. Only some mutations allow the prediction of a phenotype. In general, genotype-phenotype correlation and prediction of MPS-I patients’ clinical phenotype via genetic analysis is difficult,

due to the high degree of genotypic heterogeneity and to the wide clinical variability in patients population⁶⁹. IDUA activity in normal human fibroblasts varies in a wide range, and non-pathogenic allelic variants (polymorphisms) giving a low rate of residual activity have been reported, that are likely to modify the severity of MPS-I disease when combined with known pathogenic mutations⁷⁰⁻⁷². As little as 0.4% of residual activity is sufficient to produce a mild phenotype, while all forms of MPS-I-H have nearly undetectable enzyme activity with current techniques, which are not able to detect small differences in residual enzyme activity. For this reason, residual enzyme activity cannot be used to predict disease phenotype⁷³.

2.5 Clinical manifestations of MPS-I

The lack of HS and DS degradation in MPS-I patients results in their progressive accumulation in the lysosomes and in the extracellular matrix. The subsequent abnormalities in cell function, as well as in the structural support to the extracellular matrix, cause progressive multi-organ dysfunction and damage.

MPS-I is characterized by a marked clinical heterogeneity. The majority of patients falls into the severe group, the best described and more precisely defined. The cases of attenuated MPS-I, instead, are more various in term of age of onset, presenting symptoms, clinical signs and rate of disease progression⁷². Nonetheless, a gradual multisystemic deterioration is typical of all MPS-I subtypes. *Table 1* summarizes the main clinical manifestations of the three MPS type I

variants (MPS-I-H, MPS-I-H/S, and MPS-I-S), while a detailed description of MPS-I-H symptoms is provided below^{56,72,74-76}.

	“Severe” MPS I, MPS IH	“Intermediate” MPS I, MPS IHS	“Attenuated” MPS I, MPS IS
General	Early (<12 months) onset, rapid disease progression, hepatosplenomegaly, hernias (inguinal, umbilical, and hiatal), and death in first decade if untreated	Intermediate onset, hepatomegaly, and hernias (inguinal, umbilical, and hiatal)	Childhood onset, hernias (inguinal, umbilical, and hiatal), and normal life expectancy
Cognition	Normal early development, developmental delay/plateau, and neurocognitive decline	Learning disability possible and attention deficit possible	Typically no symptoms
Neurologic	Communicating hydrocephalus	Cervical spinal cord compression and cervical instability	Cervical spinal cord compression and cervical instability
Ophthalmologic	Corneal clouding and open-angle glaucoma	Corneal clouding and open-angle glaucoma	Corneal clouding, open-angle glaucoma, and retinal degeneration
Otolaryngological	Chronic recurrent rhinitis, persistent nasal discharge, obstructive sleep apnea, recurrent acute otitis media, and mixed hearing loss	Chronic recurrent rhinitis, persistent nasal discharge, obstructive sleep apnea, recurrent acute otitis media, and mixed hearing loss	
Cardiac	Valvular dysplasia and insufficiency, cardiomyopathy Cor pulmonale (especially with sleep apnea), myocardial infarction	Valvular dysplasia and insufficiency Cor pulmonale (especially with sleep apnea)	Valvular dysplasia and insufficiency
Orthopedic	Vertebral dysplasia, Kyphosis/lumbar gibbus, hip dysplasia/dislocation, global restriction of joint mobility, carpal tunnel syndrome, short stature, and osteopenia/osteoporosis	Vertebral dysplasia, kyphosis/lumbar gibbus, hip dysplasia/dislocation, global restriction of joint mobility, Carpal tunnel syndrome, short stature, and osteopenia/osteoporosis	Lumbar spondylolisthesis, lumbar spinal compression, joint stiffness, Carpal tunnel syndrome, milder short stature, and osteopenia/osteoporosis

Table 1. Symptoms of the three subtypes of MPS-I. MPS-I-H, MPS-I-H/S, and MPS-I-S⁷³.

General considerations. MPS-I patients usually appear normal at birth. In the first months of life, growth is often excessive. The most frequent signs at clinical presentation are coarse *facies*, movement difficulties, corneal clouding, respiratory infections, umbilical hernia, skeletal deformities, and movement difficulties, that begin before the first year of life. At about 2 years of age, growth starts to delay. Without appropriate treatment, Hurler patients’ life expectancy is severely limited: the median survival is less than 5 years. Death usually occurs for cardiac and respiratory complications.

Cognitive development and neurologic manifestations. One of the main signs of Hurler syndrome is a progressive cognitive impairment.

Early development is normal, but a delay becomes obvious between the first and second year of life, then patients undergo a progressive neurocognitive decline. Language skills are minimal because of enlarged tongue, hearing loss and cognitive impairment. Communicating hydrocephalus and chronic increases in intracranial pressure are frequent. Spinal cord compression and cervical instability are often observed.

Ophthalmologic manifestations. Some degree of visual deficit and corneal clouding is observed in all patients.

Ear, nose and throat manifestations. Chronic recurrent rhinitis and ear infections affect almost all patients, often requiring tonsillectomy and adenoidectomy or even T-tube placement in order to facilitate breathing. Obstructive sleep apnea is typical. Conductive and neurosensory deafness is common. Hearing loss can also occur due to ear infections or defective ossification of middle-ear bones.

Respiratory manifestations. The risk of severe respiratory insufficiency due to obstructive sleep apnea or restrictive lung disease is high. Restrictive lung disease from skeletal abnormalities of the chest and spine and hepatosplenomegaly can occur.

Cardiac manifestations. The typical cardiac manifestation of the disease is valvular dysplasia, with mitral and aortic regurgitation. Arrhythmia, cardiomyopathy, congestive heart failure, and coronary artery disease are common.

Abdominal manifestations. Organomegaly for GAGs accumulation, in particular liver and spleen enlargement, is frequent among patients, and it causes abdominal protuberance. Recurrent inguinal and umbilical hernias are also common.

Skeletal manifestations and joint disease. Skeletal complications are the hallmark of MPS-I, as well as of the other Mucopolysaccharidoses. The term “dysostosis multiplex” sums up the spectrum of bone abnormalities that characterize these disorders. Deficient GAGs metabolism alters both endochondral and intramembranous ossification, and GAGs infiltrations into tendons, ligaments, and joints impairs mobility. All patients develop bone and joint disease in a progressive form, and its gradual worsening leads to joint stiffening and arthropathy, and ultimately to significant movement impairment and disability. The following skeletal defects have been described in patients: dorsal gibbus, flattened and beaked vertebrae, scoliosis and kyphosis, macrocephaly, J-shaped sella turcica and platyspondyly, atlanto-occipital instability, dental abnormalities, narrow pelvis, flared iliac wings, hips dysplasia or subluxation, thick ribs and short thick clavicles, metaphyseal and diaphyseal alterations, valgus and varus deformities, *genu valgum*, bullet-shaped phalanges with typical claw hands deformity and trigger digits. Osteopenia, osteoporosis, and microfractures cause generalized pain.

Figure 2 and Figure 3 illustrate the main phenotypic manifestations and the typical bone abnormalities of the disease, respectively.

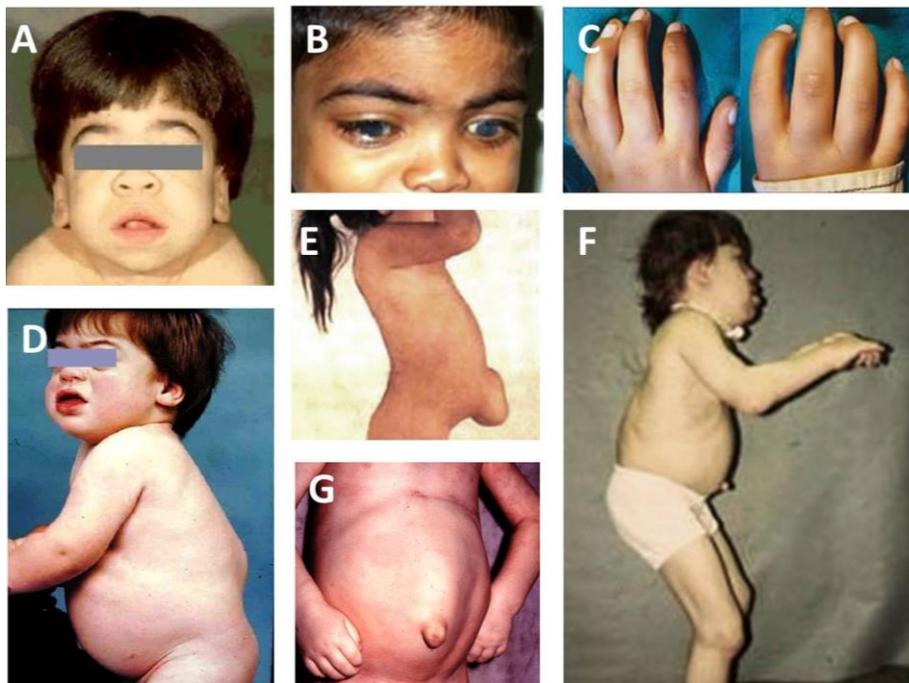


Figure 2. Common phenotypic manifestations of Hurler syndrome. A) Coarse facial features. **B)** Hirsutism, corneal clouding. **C)** Claw hands. **D)** Macrocephaly, dorsal gibbus, prominent abdomen. **E)** Umbilical hernia. **F)** Joint stiffness, genu valgum. **G)** Protruding abdomen due to organomegaly (mps1disease.com).



Figure 3. Common skeletal manifestations of Hurler syndrome (dysostosis multiplex). **A)** Macrocephaly, J-shaped sella turcica (arrowhead). **B)** Atlanto-occipital instability. **C)** Dorsal gibbus. **D)** Cardiomegaly (white arrow), thick ribs (arrowheads), short thick clavicles (black arrow) and scoliosis. **E)** Metaphyseal and diaphyseal alterations. **F)** Bullet-shaped phalanges. **G)** Bilateral calcaneovalgus deformities of feet. **H)** Genu valgum. **I)** Narrow pelvis and flared iliac wings^{75,77}.

2.6 Diagnosis of MPS-I

Even if LDs are usually caused by single enzyme deficiencies, their diagnosis is challenging, because they present a spectrum of manifestations that only partially depends on the amount of residual enzyme activity. Age of onset, symptoms severity, affected organs, and life expectancy can vary markedly. Even though some known mutations are associated with certain phenotypes and outcomes, genotype-phenotype correlation is typically not strong. All these factors limit the current diagnostic capacity⁷³.

The MPS-I Registry – an international observational database created in 2003 to characterize the natural history, long-term follow-up, and treatment outcome of the disease – offers information about MPS-I diagnostic trends. Despite MPS-I-H babies often show the very first disease signs before the 6th month of life (fail newborn hearing screen, respiratory symptoms, difficulty latching, and otitis media), the suspect of MPS-I typically derives later from clinical signs and symptoms that indicate an already advanced state of the disease: changes in facial features, limited joint movement, skeletal deformities, large head circumference and frequent respiratory infections^{78,79}. MPS-I diagnosis is based on biochemical markers such as primary storage material (DS and HS) in biological fluids (urine, serum or plasma, and liquor), or IDUA activity deficit in leukocytes, cultured fibroblasts or plasma. Diagnosis confirmation relies on molecular analysis, which allows the definition of the mutation by sequencing of the *IDUA* gene^{72,80-82}.

The median age of MPS-I-H symptoms onset is 6 months (range between 0 months and 6.5 years), the median age at diagnosis is 10

months (range between 0 months and 23.8 years), and the median age of first treatment is 17 months⁷⁸. This attests a considerable delay between MPS-I-H age of onset and age of diagnosis, and further time is required for diagnosis confirmation, treatment decision, and treatment initiation. This limits the effectiveness of the currently available therapeutic options for the disease (enzyme replacement therapy and hematopoietic stem cell transplantation), which successful outcome depends on patients' age at treatment start⁸³⁻⁸⁵. Indeed, by the time a therapy is begun, the substantial progression in the storage of GAGs has often caused irreversible damages.

Since early diagnosis and treatment can significantly prevent disease severity, associated disabilities and death, MPS-I and other LDs have been recognized as ideal candidates for newborn screening (NBS) programs. Indeed, in February 2016, MPS-I was added to the recommended panel for newborn screening by the US Secretary of Health and Human Services⁸⁶. NBS is a preventive public health program for perinatal identification of congenital disorders that can affect newborns long-term health. These disorders are selected for NBS on the basis of their prevalence, treatment availability, outcome and overall cost effectiveness⁸⁷. NBS for MPS-I and other MPSs can be realized from neonatal dried blood spots (DBSs). A few drops of blood are collected from newborns' heel between the second or third day of life, put on a filter paper, and allowed to dry. The sample is then analyzed by tandem mass spectrometry to verify GAGs content or, more frequently, enzyme activity (*Figure 4*)^{88,89}. In the last few years, the sensitivity, specificity and cost effectiveness of these assays have been markedly improved, and MPS-I NBS by determination of IDUA

activity in DBSs is currently underway in the US, and in pilot programs in Taiwan, Italy, Austria, and Hungary⁸⁹.

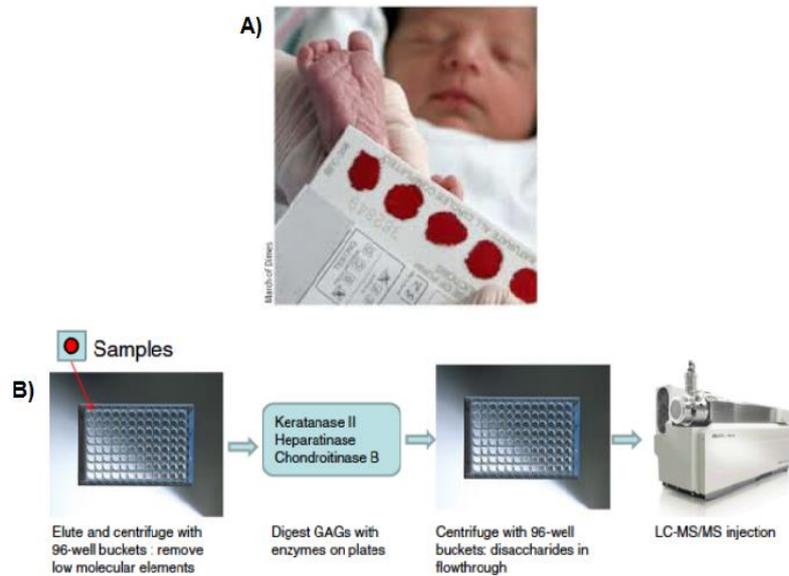


Figure 4. Newborn screening process. A) Spots of neonatal blood are deposited on filter paper. B) Sample preparation for screening by tandem MS⁵⁵.

Prenatal diagnosis is also available, based on the measurement of either enzyme activity or GAGs content in amniotic fluid cells, chorionic villi or cord blood, or molecular genetic testing in families where the mutations has already been identified^{90,91}.

2.7 Murine models of MPS-I

Up to now, six MPS-I murine models have been described and employed to investigate the pathological mechanisms and the possible therapeutic approaches in this disease.

Four immunocompetent models on a C57BL/6 background were generated independently by knock-out⁹²⁻⁹⁴ or knock-in⁹⁵ of the murine *Idua* gene, that maps on chromosome 5⁹⁶. Two models are, instead, immunodeficient: one is on a NOD/SCID background⁹⁷, and one on an NSG background⁹⁸.

Clarke MPS-I model. The first of these models was generated in 1997 by Lorne A. Clarke *et al.* by targeted disruption of the *Idua* gene in R1 murine embryo stem cells⁹². Due to the presence of an overlapping gene (*Sat-1*), the authors disrupted the *Idua* gene with a construct directed to exon VI, and they used an interruption type construct rather than a deletion one. Chimeric mice were mated with C57BL/6 animals, and a colony of *Idua*^{+/+}, *Idua*^{+/-} and *Idua*^{-/-} (MPS-I) mice was established through heterozygote cross.

Idua disruption results in a complete deficiency of alpha-L-iduronidase activity in all the tissues. Urine GAGs excretion is on average 3-fold more than the normal counterparts. The phenotype of these mice has been extensively characterized in this and further works^{92,99-102}. Young affected mice are active and capable of a full range of movements, but with age they show a progressive decline in mobility, and they develop gibbus deformity and subluxation of the hips. No difference in mortality is seen in the first 20 weeks of life, indicating that the phenotype is somewhat attenuated in this murine model. Anyway, MPS-I mice clearly have a shortened lifespan, because their average age of death is 48 weeks, while amongst control littermates only about 5% dies of natural causes within the first 90 weeks.

Concerning the growth profile, affected animals grow at or above the 50th percentile from 8 to nearly 25 weeks of age, then their growth reaches a *plateau* and becomes comparable to the siblings' one.

At birth and during the first 2-3 post-natal weeks, there are no macroscopical differences between *Idua*^{-/-} mice and their littermates. Yet, by 4 weeks of age, progressive craniofacial dysmorphisms with altered facial features becomes discernible, particularly in males: affected mice have broadened cranium, coarse face, loss of the fine tapered snout, foreshortened snout, protruding nasal bridge, redundancy of the periocular tissue, flattened facial profile. Differences in the morphology of the paws are evident: the paws of MPS-I mice are broadened, and the digits and the palmar regions thickened (*Figure 5*).

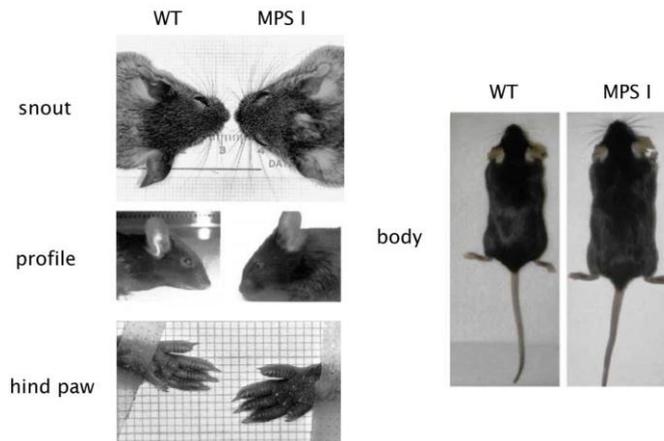


Figure 5. Phenotype of the MPS-I model. Photographs of WT and MPS-I mice aged 12 weeks, showing the appearance of their snout, hind paws, and body^{92,100}.

The cardiovascular abnormalities found in these mice include aortic root dilatation, aortic valve insufficiency, and decreased ventricular function.

Radiographs reveal features of skeletal dysplasia with early dysostosis multiplex, starting from 4 weeks of age and progressively worsening: enlargement of the cranium, coarseness of the facial bones with thickening of the zygomatic arches, widening of the vertebrae, thoracic vertebral kyphosis, widening and increased density of the ribs, broadened and sclerotic long bones, general thickening of the diaphysis of the long bones, significant increase in mid-femur diameter, and decrease in tibia and foot lengths (*Figure 6*). Micro-CT analysis shows anomalies in both the cortical and trabecular portion of the femur, abnormal periosteal bone formation in the knee joint and thickened posterior vertebral arches of cervical vertebrae, with thickened periosteum in the bones of the knee joint and in cervical vertebrae, as well. These anomalies are initially more pronounced in males, but by 16 weeks of age the gender difference is less apparent.

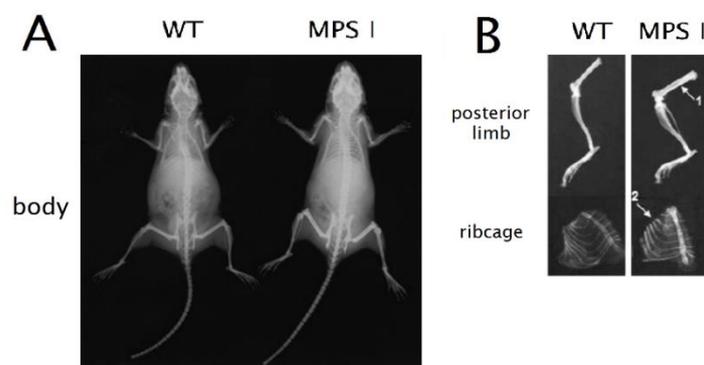


Figure 6. Skeletal phenotype of MPS-I mice. **A)** Full body radiographs of 15-weeks-aged mice show general thickening of the bones, coarseness of the facial bones, and widened ribs in the affected. **B)** At 11 months, affected mice show sclerotic femur and “oar-like” ribs^{92,100}.

Neufeld MPS-I model. In 2003, a new MPS-I model was generated by the research group of E. Neufeld, again by targeted disruption of the exon VI of the gene in a C57BL/6 inbred background⁹³. The phenotype of these animals is similar to the one of Clarke's model^{93,103-108}.

Immunodeficient MPS-I models. Regarding the immunodeficient models of MPS-I, less likely to develop immune reactions in case of IDUA enzyme infusion or transplantation of human cells, in 2007 Garcia-Rivera and co-workers developed a NOD/SCID MPS-I model⁹⁷. These mice exhibit undetectable IDUA activity, marked GAGs accumulation in tissues and urinary GAGs excretion, and limited motor skills. Their skeletal phenotype is not well characterized, but it is milder than the one of Clarke's model. They are short-lived, so they provide only a narrow window to assess the long-term efficacy of any therapy. Also, since they develop thymic lymphomas, they are not a good model for the assessment of potential tumorigenicity of human stem cell transplantation. Thus, another model was developed in 2015 by Mendez *et al.*, on a NOD/SCID/IL2R γ (NSG) background⁹⁸. The life-window of this model is longer than 1 year, and mice are tumor-free during that time. IDUA is undetectable in tissues, while GAG levels are high. They show coarsened facial features, reduced gait, and kyphosis, but their skeletal abnormalities have a slow development and they have not been described in detail.

Skeletal disease mechanisms: lessons from MPS-I mice. Although MPS-I is associated with skeletal abnormalities, the impact of IDUA deficiency on bone remodeling is poorly defined: the mechanisms underlying the joint and skeletal tissue abnormalities distinctive of

MPSs are under investigation in these models. Probably GAGs storage, determining loss of cellular function and triggering a cascade of secondary pathological effects, is responsible for tissue damage and dysfunction, which leads to the abnormalities observed in patients¹⁰⁹.

The skeletal disease of MPS-I mice is a result of disturbed endochondral ossification, intramembranous ossification and bone remodeling. As described above, morphogenic abnormalities have been noticed in the cortical bone and, less markedly than in other MPSs, in the growth plate. In particular, in all the bones of affected animals, a generalized thickening of the cortical region is evident, probably due to a perturbation in the parallel lamellar organization and to a persistence of islands of unossified cartilage^{99,100,109}.

The basis of these ossification defects needs to be investigated. Impairment in the function of osteoclasts, the cells deputed to cartilage resorption during the ossification process, seems to be the main responsible mechanism. For example, Kuhen *et al.* showed that MPS-I mice progressively develop a high bone mass phenotype, and their osteoclastogenesis parameters are significantly reduced. They state that, since osteoblasts in affected mice are not only affected by lysosomal storage, but also numerically decreased, the bone mass increase in untreated MPS-I mice could be explained by impaired osteoclast differentiation and/or function. Also, since 6-week-old MPS-I mice do not display the abnormalities found in older animals, both bone overgrowth and high trabecular bone mass should be the consequence of altered bone remodeling, and not of impaired skeletal development¹⁰². Other works confirm the role of impaired osteoclastogenesis in MPS-I and identify some of the molecular

mechanisms that could be involved. It has been demonstrated that DS and HS directly inhibit cathepsin K, a lysosomal protease highly expressed by osteoclasts and essential for the degradation of collagen type II, the principal component of cartilage¹¹⁰. Our group has demonstrated that MPS-I human mesenchymal stem cells show alterations in the OPG/RANK/RANKL pathway, that is involved in several inflammatory and bone diseases: this could be another mechanism involved in the disease¹¹¹.

For what concerns MPS-I joint disease, many studies agree that inflammation following GAGs accumulation is one of the main triggers of articular cartilage anomalies, and matrix metalloproteinases (MMPs), inflammatory cytokines and inflammatory cells such as macrophages have been observed within MPS-I mice joints^{108,112-115}. The inflammatory state and the activation of macrophages exacerbate chondrocytes death and consequently joint degeneration. The alterations of joint cartilage in MPS-I mice worsen with age, manifesting with progressive depletion of the components of the extracellular matrix (collagen, proteoglycans, elastin), fibrocartilagineous proliferation, irregularities at the articular surfaces, cartilage resorption and ultimately bone resorption.

3. TREATMENT OF MPS-I

The better understanding of disease pathogenesis and natural history, and the advancing availability of both supportive and disease-specific therapies have improved the outlook for MPS patients during the last decades. It is unanimously agreed that, given the progressive nature of these disorders, early diagnosis and, most of all, early treatment are of major importance^{85,116-118}. Disease progression gives rise to worsening organ damage, a secondary effect of GAGs accumulation, which is often irreversible. Hence, treatment response depends on the severity of the disease phenotype and on the degree of disease progression at treatment initiation.

Diagnosis, treatment choice and fulfilment, and patients follow-up require a multi-disciplinary approach, since MPSs usually present a broad and complex spectrum of manifestations. Before the advent of MPS-specific therapies, symptom-based interventions were performed to improve patient's lifespan and quality of life, and to reduce the secondary complications of the disorders^{27,116}. They are still employed nowadays as supportive therapies, and the mainly consist in surgical interventions (such as shunt for communicating hydrocephalus, corneal transplant for corneal clouding, tonsillectomy and adenoidectomy or tracheostomy for airway obstruction, cardiac valve replacement for valve regurgitation, median nerve release for carpal tunnel syndrome, hernia repair), spinal fusion for spinal cord compression and other orthopaedic procedures to correct skeletal defects, physical therapy to minimize joint contractures and stiffness, and speech and behavioural therapy to ameliorate developmental skills.

In the last few decades, a deeper comprehension of the physiopathological mechanism at the basis of MPSs was achieved thanks to the advances in scientific research. Thus, disease-specific treatments were developed, to solve the deficit by supplying the missing enzyme to the body.

In 1968, Fratantoni and Neufeld demonstrated that fibroblasts from patients with Hurler syndrome and Hunter syndrome (Mucopolysaccharidosis type I and II), when cultured together, compensated each other's defect, and both cell types acquired the ability to degrade GAGs²⁵. The mechanism by which cells complemented each other's defect was the exchange of the necessary "factors", the defective enzymes, through the culture medium. This phenomenon was called "cross-correction", the process by which the proximity of normal cells leads to the correction of the biochemical consequences of enzymatic deficiency within surrounding cells²². The cross-correction of MPS cell defect is possible because, as described above in this thesis (paragraph 2.1 Lysosomal diseases), a minority of the M6P-tagged lysosomal hydrolases escapes lysosomal targeting, and is secreted in the extracellular space. Thus, the enzymes can be recognized for their M6P-tag by the surface M6P receptors of neighbouring cells and can be uptaken by endocytosis. In this way, an enzyme-defective cell can capture exogenous enzymes and partially restore its ability to degrade GAGs⁴⁶.

The mechanism of cross-correction lies at the basis of the principal therapeutic approaches that have been developed for MPS-I and other LDSs, and that are currently employed: enzyme replacement therapy, hematopoietic stem cell transplantation, and gene therapy. Additional

small-molecules therapies based on other molecular mechanisms, such as substrate reduction, molecular chaperones, and non-sense suppression, are also under investigation.

3.1 Enzyme replacement therapy

Enzyme replacement therapy (ERT) is a medical treatment which replaces an enzyme that is deficient or absent in the body by its periodic administration to a patient.

The possibility of treating LDs by the replacement of the faulty enzyme by supplying the normal one was suggested as early as in 1966¹¹⁹. In 1971, it was confirmed *in vivo* by experiments in which Hurler and Hunter patients were treated with the administration of human plasma from healthy individuals – thus containing a quote of secreted lysosomal enzymes – obtaining a transient recovery of enzyme activity¹²⁰.

The effectiveness of ERT relies on the biodistribution of the therapeutic enzyme, that can spread throughout the body via blood circulation. The enzyme can fulfill its function in the extracellular fluids and spaces or it can be taken up by cells through receptor-mediated endocytosis and restore lysosomal function^{46,121}.

MPS-I was the first MPS treated by ERT. The enzyme became available for a potential clinical use in 1994, when Kakkis *et al.* produced and purified a polymorphic variant of human alpha-L-iduronidase by recombinant DNA technology in the Chinese hamster ovary (CHO) cell line. Recombinant IDUA was efficiently endocytosed by Hurler

fibroblasts through a M6P-dependent mechanism and it was corrective for abnormal GAGs accumulation¹²².

The first clinical trial for the use of ERT in MPS-I patients began after some preclinical studies in the canine model^{123,124}. In 2003, after the phase I/II trial, recombinant human alpha-L-iduronidase (laronidase, Aldurazyme®, Genzyme/Biomarin) was approved for the treatment of MPS-I patients. The purified enzyme is available in vials containing 5 mL of drug solution at a concentration of 0.58 mg/mL. The recommended dose is 0.58 mg/kg body weight, and it is dispersed in isotonic saline and infused over 2-4 hours, once a week.

The overall results of the clinical employment of laronidase in treating MPS-I are encouraging, since many aspects of the disease are stabilized or reversed during long-term therapy. ERT, indeed, induces a decrease in urinary GAGs excretion and in lysosomal storage of GAGs, with a significant decrease in hepatosplenomegaly, sleep apnea and hypopnea episodes, and an increase in the range of motion of shoulder and elbow, and in the rate of growth in height and weight in prepuberal patients¹²⁵⁻¹²⁸.

However, the standard ERT approach for MPS-I has some limits: laronidase has no effects on some disease manifestations, which remain stable or worsen during the treatment period. First, the intravenously infused enzyme is not expected to cross the blood-brain barrier in appreciable amounts at the administered dose. For this reason, laronidase treatment is recommended only for the attenuated forms MPS-I, with null or limited neurological involvement^{27,86,118}. Attempting to overcome this problem, by intrathecal, intranasal or brain-targeted ERT approaches, are under development¹²⁹⁻¹³⁵. Also, the

enzyme can hardly spread to organs that have a poor vascular supply, such as bone, and the correction of GAGs storage in the growth plate and in the articular cartilage is challenging, because the therapeutic enzyme diffuses slowly through the molecular structure of the matrix^{57,114,118}. Corneal clouding and valvular disease are other manifestations hardly curable by ERT.

Furthermore, in nearly all (>90%) patients undergoing ERT, immune reactions with the development of IgG antibodies anti-IDUA are detected. This does not seem to affect the clinical efficacy of the therapy, but the long-term impacts of the phenomenon are still unknown and under investigation^{127,136}.

Finally, ERT is a chronic treatment: the need for weekly infusions over a lifetime leads to compliance problems and impairs patients' quality of life. Patients' families and/or the sanitary system are charged for the high costs of individuals undergoing ERT: the cost of laronidase-based therapy is between 250000 and 1000000 dollars per year per adult patient¹³⁷.

3.2 Hematopoietic stem cell transplantation

The goal of HSCT as a therapy for LSDs is to obtain the repopulation of patient's hematopoietic compartment by metabolically correct donor cells, that represent a source of functional enzyme for the recipient organism¹³⁸. The rationale for the potential usefulness of HSCT in the treatment of MPSs relates on the demonstration of the cross-correction phenomenon by Fratantoni and Neufeld²⁵. A few years later, in 1971, a restoration of deficient enzyme activity was obtained in vivo

administering human lymphocytes to a child with Mucopolysaccharidosis type II¹³⁹.

The cross-correction realized in a MPS patient after HSCT relies on the migration, homing and engraftment of donor-derived cells to affected organs. Donor's hematopoietic cells can settle in non-hematological organs and specialize, living in close proximity to recipient's enzyme-deficient cells of these organs and releasing M6P-alpha-L-iduronidase moieties. The latter can be uptaken by surrounding cells, which corrects aberrant intracellular GAGs storage. Furthermore, the secreted enzyme can also act in the extracellular spaces, removing GAGs deposits from the extracellular matrix, and it can reach distant organs through blood and lymph circulation (*Figure 7*)²³.

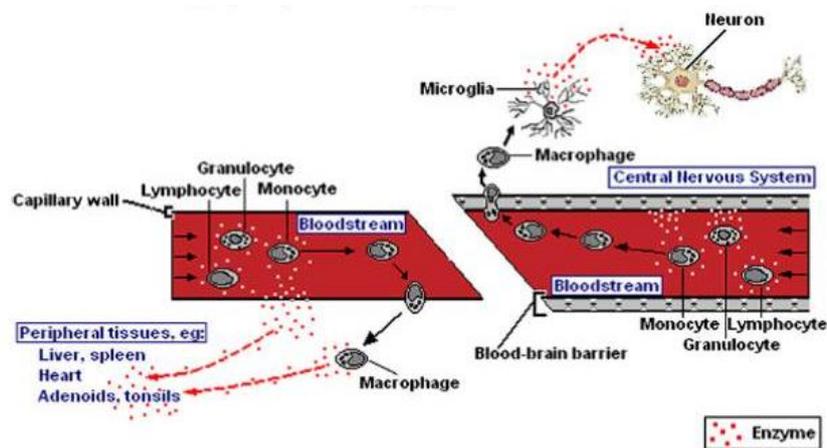


Figure 7. Enzyme delivery to peripheral tissues and central nervous system (CNS) after HSCT. From the bloodstream, the enzyme reaches the tissues, either by diffusion of the plasma enzyme, secreted by donor leukocytes, or by leukocytes leaving the circulation and entering the tissues. In the CNS, the enzyme is delivered by the latter mechanism only, because the blood-brain barrier does not permit the diffusion of the plasma-derived enzyme²⁰.

The first successful HSCT in a patient with MPS-I was performed by Hobbs *et al.* in 1981: a 1-year-old boy with Hurler syndrome was given a bone marrow transplant from his mother. A few months after graft, IDUA activity was detected in the serum and urine. Thirteen months after graft, hepatosplenomegaly and respiratory problems disappeared, growth restarted, developmental deterioration arrested¹⁴⁰.

More than 500 people affected by MPS-I have been treated with HSCT worldwide. A huge portion (approximately 45%) of the HSCTs for the cure of inborn error of metabolism has been performed in Hurler patients¹⁴¹.

The short-term and long-term follow-up of MPS-I patients after successful HSCT have been reported in many works^{20,83,84,141-147}. They attest that this therapy shows positive effects on the biochemical parameters: within 3 months from transplantation, IDUA activity in leukocytes and GAGs excretion in the urine reaches the values expected for normal individuals. This reflects on the organs: a decrease of GAGs storage in liver, spleen, heart and lungs is observed. Within the first 3-6 months after transplantation, hepatosplenomegaly reduces, and relief of airway symptoms is seen. Myocardial function is preserved and hypertrophy regresses.

Concerning the central nervous system (CNS) and neurocognitive function, donor-derived cells can repopulate the microglia and allow the reduction of GAGs storage in brain, the stabilization of communicating hydrocephalus and the prevention of the severe neurological manifestations of Hurler syndrome, with a favourable neurocognitive and motor development. This outcome is especially marked when HSCT is performed early in the course of the disease,

when intellectual function is still relatively normal and extensive cerebral damage has not occurred yet^{148,149}. HSCT, indeed, is rather recommended for patients affected by Hurler syndrome, the form of MPS-I with severe neurocognitive involvement, because it is currently the only therapy that offers benefit for central nervous system involvement. Consistent evidence from the literature suggests that transplantation in the first year of life is associated with improved developmental and intelligence quotient, and continued cognitive growth, with earlier age of treatment associated with improved outcomes⁸⁶.

A reduction of joint pain and improvements in the range of motion of upper extremities is documented, while the outcome for the lower extremities is less positive¹⁵⁰. Linear growth is maintained for years after HSCT, but it stunts in long-term follow-ups. Overall life expectancy after HSCT is significantly improved, and extends into the third decade of life, mainly as a result of the prevention of cardio-pulmonary complications.

However, some disease-associated problems are hardly corrected by HSCT. Indeed, heart valves deformities and thickening persist and often progress, leading to the requirement of valves substitution interventions. Corneal clouding degree often does not regress, progressive retinal degeneration and optic atrophy continue, thus the visual acuity of long-term surviving patients continuously worsens^{83,146}. The repopulation of microglia by donor cells happens more slowly than in other tissues (the process requires approximately one year), so the neurocognitive abilities in the immediate post-transplantation period

continue to decline and HSCT may be unable to stabilize or prevent neurologic deterioration in the most rapidly progressing cases^{148,149}.

Finally, but importantly, the framework of dysostosis multiplex that characterizes MPS-I patients receives limited benefit from HSCT: carpal tunnel syndrome, thoracolumbar kyphosis, hip subluxation, fixed flexion deformities of the lower limbs and *genu valgum* frequently show progression. Again, patient's age at transplantation seems to be an important variable for the skeletal outcome: if HSCT is performed before the skeletal anomalies have reached an advanced, irreversible state, it shows a certain degree of efficacy in preventing and delaying musculoskeletal degeneration^{117,150}.

Other limitations of this approach are, instead, relative to the transplantation procedure itself^{83,141,151}. Acute and/or chronic GVHD, pulmonary complications and immunocompromisation are the main transplantation-related complications, mainly determined by the conditioning regimen. Unsuccessful engraftment after the first transplantation occurs with a frequency that varies between 34% and 85%, especially in patients subjected to T-cell depletion and reduced-intensity conditioning. At least 20-25% of Hurler patients needs a second graft, which rate of success is actually high, around 80%. The overall survival rates of patients after HSCT is about 85%.

Currently, UCB is the most attractive source of cells for HSCT in MPS-I, especially for infant patients, because it displays several benefits (see paragraphs 1.2 and 1.3)^{17,19,22,83,144}. Cord blood immune cells still have a certain degree of immaturity, thus the tolerable HLA disparity is higher in UCBT than in HSCT from BM or peripheral blood. Due to more permissible HLA mismatch, an UCB donor can often be found

rapidly, and this is an advantage where rapid treatment leads to better outcome. Moreover, UCB has been found to lend both greater engraftment and higher enzyme levels compared to the other sources of HSCs.

3.3 Gene therapy

MPS-I, like many other LSDs, is an ideal candidate for gene therapy, since it is a single-gene disorder, the defective gene is well characterized, the enzyme is not subjected to complex regulation mechanisms, and little enzymatic activity is sufficient for a clinical improvement⁸⁷. By the means of gene therapy, organs or cells could be modified to stably secrete physiological or supra-physiological levels of functional IDUA enzyme. The aim of gene therapy is, in fact, the delivery of a normal copy of the defective gene to patient's cells, that thus become able to synthesize the functional protein and to act as a source of enzyme in the body. The genetic modification can be obtained employing viral vectors or other gene transfer agents^{53,152}. Gene therapy approaches can be realized:

- *in vivo*, injecting the viral transfer vectors sistemically, directly into the bloodstream or tissues
- *ex vivo*, modifying HSPCs or other stem cells, and then transplanting them into the recipient.

***In vivo* gene therapy.** *In vivo* gene therapy approaches are realized by the administration to the patient of either lentiviral vectors (LV), retroviral vectors (RV), or adeno-associated viral vectors (AAV). These

approaches are demonstrated to provide benefits thanks to the achievement of sustained and robust enzyme production by transduced cells. However, their efficacy is limited by the occurrence of immune responses and by a poor bio-distribution of the enzyme secreted in the blood¹⁵³⁻¹⁵⁶. Indeed, in the case of MPS-I, these studies show few benefits at the CNS level, and little, if any, long-term beneficial effect on the disease manifestations after systemic injection of RV or LV encoding the functional IDUA in adult MPS-I mice. This failure was likely due to a transgene-specific immune response, leading to the clearance of transduced cells¹⁵³. Chief amongst the factors contributing to the induction of an immune response following gene transfer is the direct expression of the transgene product within professional antigen-presenting cells of the immune system¹⁵⁷.

***Ex vivo* gene therapy.** Different cell types at diverse stage of maturation could be targeted by *ex vivo* gene therapy vectors and administered to LD patients as therapeutic agents providing the deficient enzyme. The most interesting target cells for this kind of approach are HSCs¹⁵². HSCT represents a reliable therapeutic opportunity for MPS-I and other LDs, but its usefulness is limited by the need for HLA-matched donors, the morbidity and mortality associated with the transplant procedure, the low amount of enzyme that transplantation can provide to an entire organism, and the suboptimal therapeutic impact on the CNS and skeletal manifestations. Patients' clinical outcome could be further ameliorated combining HSCT with gene therapy^{118,152}. HSC-based gene therapy approaches aim at conferring HSCs the ability to produce a functional form of the

defective enzyme, and then reintroducing them into the organism by an autologous HSCT, obtaining physiological or supraphysiological levels of enzymatic activity in biological fluids¹⁵⁸.

Among the different vectors that could be employed to deliver a copy of the gene of interest to defective HSCs, it has been demonstrated that lentiviral (LV) vectors could meet the requirements for sustained enzyme expression upon HSCs transduction. Also, LV vectors could lead to robust gene expression in HSCs progeny and are characterized by a good safety profile, with minimal risk of insertional mutagenesis^{159,160}. LV HSC gene therapy has already received marketing authorization for the treatment of severe combined immunodeficiency caused by adenosine deaminase deficit (ADA-SCID), in April 2016. Clinical trials using LV-transduced HSCs are ongoing in patients with adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), β -thalassemia, and Wiskott-Aldrich syndrome (WAS), and the trial on MPS-I is expected to start in January 2018. So far, it has been demonstrated that autologous LV HSC gene therapy for these diseases can^{158,160}:

- exploit the unique properties of HSCT, capable of repopulating affected tissues (including the CNS) with myeloid cells delivering the functional enzyme
- improve the therapeutic potential of HSCT by enzyme overexpression in myeloid cells
- reduce allogeneic HSCT side effects and morbidity/mortality.

In the specific case of LDs, the efficacy of LV HSC gene therapy in controlling disease manifestations has been tested in MLD and MPS-I^{107,152,158,161-165}. For what regards MPS-I, using LVs to efficiently

transduce murine BM HSC, Visigalli *et al.* proved the potential of their progeny to efficiently target IDUA gene to multiple tissues. The authors successfully modified MPS-I BM cells with a IDUA ubiquitously expressing LV vector, and achieved supra-physiological levels of enzyme activity that guaranteed a therapeutic benefit on the neurologic and skeletal anomalies, critical disease manifestations¹⁰⁷. In their subsequent work, in order to support the clinical application of LV HSC gene therapy in MPS-I, biosafety studies were conducted to assess the toxicity and tumorigenic potential, as well as the biodistribution of HSPCs transduced with IDUA-expressing LV vector, and vector integration site studies were applied in order to predict the adverse consequences of vector gene transfer¹⁶⁵. The obtained data provided a strong rationale for testing this therapy in MPS-I patient. Indeed, LV vectors may provide the opportunity of achieving high IDUA expression levels in HSCs and their differentiated progeny, and therefore efficacious metabolic correction in MPS-I affected tissues, including the brain and the skeleton. Importantly, HSC gene therapy may also limit the risk of developing antibodies to the secreted enzyme as the procedure is likely to establish tolerance to the transgene (the functional IDUA) introduced into the transplanted HSC. This tolerogenic effect consequent to HSC gene therapy may be relevant also in the case of combined transplantation strategies.

Among other cell types that have been modified by *ex vivo* gene therapy in an attempt to ameliorate the symptoms of MPSs in the animal model, there are other stem cells such as neural stem cells¹⁶⁶, and committed cell types that can act as transient sources of enzyme, such as stromal cells¹⁶⁷⁻¹⁶⁹ and macrophages¹⁷⁰.

The big challenge of this kind of treatments remains the invention of a safe delivery system: beside immune reactions to the viral proteins, in fact, the real risk of gene therapy is represented by the disruption of gene integrity and regulation in the recipient^{171,172}.

3.4 Small molecules therapies

More recently, the increasing knowledge of the pathological mechanisms involved in LDs has allowed the invention of other therapeutic strategies, that are still under investigation in animal models. Substrate reduction therapy, but also molecular chaperones, and non-sense suppression therapy, seem promising possibilities, and may be developed as adjuvants of ERT or HSCT.

Substrate reduction therapy. The aim of substrate reduction therapy (SRT) for LSDs is the partial inhibition of the biosynthesis of the accumulated products, to diminish the influx of these substrates into their defective catabolic pathway. This treatment can only limit the accumulation, so it requires a certain degree of residual enzyme activity in patients or, alternatively, it can be used in combination with other therapies, such as ERT or HSCT^{173,174}. Substrate reduction can be obtained by pharmacological agents that inhibit the enzymes responsible for the production of the involved substrates. For example, in the case of MPSs, genistein, rhodamine B, and other compounds that interfere with GAGs synthesis are being studied¹⁷⁵⁻¹⁷⁹. Some of the compounds used for SRT can penetrate the blood brain barrier, so SRT could be an effective adjuvant therapy in reducing brain storage and the neurological manifestations of these diseases¹⁸⁰.

Molecular chaperones. In the secretory pathway, misfolded enzymes are often recognized by quality control systems of the endoplasmic reticulum and addressed to degradation. Chaperones are molecules that restore the native conformation of misfolded proteins under physiological conditions. As a therapeutic strategy for LSDs, small molecular chaperons can be used to improve the folding and trafficking to the lysosomes of missense mutated lysosomal enzymes, both enhancing their residual activity and avoiding their degradation. Some of these small molecules can pass the blood brain barrier and act also within the CNS^{53,180}. In particular, the molecular chaperone 1-deoxyidronojirimicin has already been preclinically evaluated for the treatment of MPS I-H/S¹⁸¹.

Nonsense suppression therapy. Nonsense mutations are frequently found in LSDs: they originate a premature stop codon, that causes the termination of translation and the generation of a truncated unfunctional enzyme. Gentamycin or its analogues like NB54 can enable the translation machinery to read through the premature stop codon, and thus to produce a full-length protein, that could be at least partially functional^{182,183}. Such drugs could have a role in treating MPS-I-H, as the majority of Hurler patients carries at least one nonsense allele in their DNA.

4. AIM OF THE THESIS

The aim of my PhD project was to develop a hematopoietic stem cell transplantation (HSCT)-based therapeutic approach for MPS-I, combining an early (neonatal) time of intervention and the use of umbilical cord blood (UCB) as a source.

The project has developed as follows:

- First, we illustrate in a review the state of the art on neonatal cellular and gene therapies for Mucopolysaccharidoses (MPSs). We provided evidence that the therapeutic approaches to MPSs should focus on the time at which the treatment is administered: the clinical outcome could be more favorable if the therapy means to prevent, instead of correcting, disease manifestations.
- Then, we present our recently published work, in which we tested the therapeutic efficacy of UCB transplantation (UCBT) in newborn MPS-I mice. We characterized the phenotypical and functional features of murine UCB cells, and we demonstrated the effectiveness of neonatal UCBT in ameliorating the biochemical and skeletal abnormalities of the mouse model.

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CHAPTER 2

Neonatal cellular and gene therapies for Mucopolysaccharidoses: the earlier, the better?

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ABSTRACT

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders (LSDs). The increasing interest in newborn screening procedures for LSDs underlines the need for alternative cellular and gene therapy approaches to be developed during the perinatal period, supporting the treatment of MPS patients before the onset of clinical signs and symptoms.

The rationale for considering these early therapies results from the clinical experience in the treatment of MPSs and other genetic disorders. The normal or gene-corrected hematopoiesis transplanted in patients can produce the missing protein at levels sufficient to improve and/or halt the disease-related abnormalities. However, these current therapies are only partially successful, probably due to the limited efficacy of the protein provided through the hematopoiesis. An alternative explanation is that the time at which the cellular or gene therapy procedures are performed could be too late to prevent pre-existing or progressive organ damage. Considering these aspects, in the last several years, novel cellular and gene therapy approaches have been tested in different animal models at birth, a highly early stage, showing that precocious treatment is critical to prevent long-term pathological consequences.

This review provides insights into the state-of-art accomplishments made with neonatal cellular and gene-based therapies and the major barriers that need to be overcome before they can be implemented in the medical community.

INTRODUCTION

Background

Mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders (LSDs) having in common the inherited deficiency of a particular lysosomal enzyme and the subsequent accumulation of undigested glycosaminoglycans (GAGs). GAG storage results in loss of cellular functions, tissue damage, and organ dysfunction accounting for clinical signs and symptoms observed in patients. Clinical manifestations include mental retardation, skeletal dysplasia, corneal clouding, abnormal facies, coarse hair, hernia, hepatosplenomegaly, respiratory and valvular heart diseases, and abnormal joint mobility¹. At the skeletal level, MPS patients develop a characteristic dysostosis multiplex due to the progressive storage of GAGs in the bones, especially chondroitin sulfate, dermatan sulfate and/or keratan sulfate¹.

Difficulty in early diagnosis

It is extremely difficult to diagnose MPS patients at birth, and even at the onset of clinical disease. For instance, most MPS I patients may not have MPS-specific signs and symptoms at birth although umbilical and/or inguinal hernia is common. Diagnosis of Hurler syndrome, the most severe form of MPS I, is commonly made between 4 and 18 months of age. A combination of symptoms, as skeletal deformities, recurrent respiratory infections, inguinal and umbilical hernias, coarse facial features, hepatosplenomegaly, and enlarged tongue leads to initial medical attention. Without appropriate treatment, the life expectancy of patients with Hurler syndrome is limited: the median

survival is less than 10 years, with only rare survivors beyond 10 years. Diagnosis of other type of MPS may be delayed more than MPS I since clinical manifestations occur later.

Early treatments

Currently, several treatments such as enzyme replacement therapy (ERT), hematopoietic stem cell transplantation (HSCT), and gene therapy are being evaluated for MPSs. ERT and HSCT are clinically available, and gene therapy is under clinical trials for some types of MPSs. ERT, based on the administration of recombinant enzyme, is actually being employed in patients with MPS I², MPS II^{3,4}, MPS IVA⁵, MPS VI⁶, and MPS VII⁷. Indeed, the treatment with ERT has shown demonstrable benefits, especially in joint mobility, respiratory functions, decrement in organs volumes, and reduction in urinary GAG excretion⁸. However, current ERT for MPSs has severe limitations, such as an inadequate effect on skeletal and neurological symptoms⁸⁻¹⁰, a rapid clearance from the circulation, and immune reactions, due to the development of anti-enzyme antibodies^{2,11}. Several demonstrations have shown that the earlier ERT is performed in animal models and human patients, the better the outcome is¹²⁻¹⁴. Similar evidence comes from the follow-up analysis of MPS I patients treated with hematopoietic stem cell transplantation (HSCT). Indeed, a retrospective analysis could demonstrate superior long-term clinical outcome for patients with MPS I when HSCT was performed early in life^{15,16}. HSCT of MPS I patients (transplanted with a median age of 16 months) improves their quality of life and neurocognitive development, albeit the therapeutic effect on bone lesions remains limited^{15,16}. The

musculoskeletal manifestations are still deteriorated and provide an impact on the quality of life in most transplanted patients with MPSs¹⁷. One possibility is due to the limited penetration of the expressed enzyme into musculoskeletal tissues¹⁸. Another possibility is that irreversible bone damage has already occurred prior to the time of the transplant. Considering the current experimental and clinical data on both the available treatment options, it is likely that an early intervention in patients with MPSs could be critical for obtaining a higher degree of correction. It is notable that MPSs may represent the ideal model for elucidating these aspects since 1) they are monogenic diseases, 2) several animal models are available, and 3) the successful restoration of even a low level of enzyme activity is expected to be sufficient to correct or improve the disease. For these reasons, MPSs are conceived as conditions suitable for the evaluation of innovative early therapeutic strategies.

In particular, this review focuses on the scientific evidence demonstrating that cellular and gene therapies in the neonatal period provide a real therapeutic perspective for MPS disorders.

NEONATAL HEMATOPOIETIC STEM CELL TRANSPLANTATION

Cellular therapy is relevant for some forms of MPSs. HSCT has been shown to be one of the most effective treatment strategies for patients with Hurler syndrome. In particular, the use of various HLA-matched hematopoietic stem cell sources (peripheral blood, bone marrow of unrelated donors or cord blood) has contributed to offer a transplantation strategy to a significant number of patients^{17,19-21}.

In general, early HSCT improves the pathology in all organs although bone lesions have a less impact compared with visceral organs. Thus, HSCT alleviates most clinical manifestations in these patients, probably due to the migration of the transplant-derived cells into organs, where they can secrete the functional enzyme and clear the lysosomal storage leading to the correction of the metabolic defect. However, the effect of HSCT on the orthopedic manifestations is limited likely by the poor penetration of the donor cells into the musculoskeletal tissues^{17,18}. It is likely that HSCT provides more circulating enzyme to bone rather than directly affecting bone by migration of the cells.

Furthermore, the recovery of the patients' skeletal phenotype produced by HSCT could be incomplete likely because bone abnormalities are irreversible at the time of the transplant. For this reason, the impact of HSCT could still benefit from further improvements, as the use of different stem cell sources and/or alternative transplant procedures. In particular, a neonatal cellular therapy approach may hold more promise, considering that it would allow preventing the progressive disease manifestations, which develop during early stage.

Several reports have evaluated if the perinatal infusion of stem cells of hematopoietic origin could ameliorate the most prominent clinical features in MPS animal models (*Table 1*). Soper et al. published a study describing a non-ablative neonatal marrow transplantation model in MPS VII mice²². Despite low-level engraftment of donor cells, MPSVII mice treated with bone marrow transplantation (BMT) in neonatal life have shown several improvements, including extension of life span, reduction of lysosomal storage in multiple tissues, and amelioration of bone parameters^{22,23}. Successive neonatal BMT procedure in the MPS VII mice also proved that the aberrant electrocardiogram and many of the progressive heart lesions were corrected in the long-term²⁴. Furthermore, BMT in newborn MPS VII mice has led to the prevention of early hearing loss and to an improvement in the histopathology of the ear²⁵. In the central nervous system (CNS), it has been reported a consistent reduction in the amount of storage in the meninges and glial cells, but not in the neocortex, hippocampus, and cerebellum²². It is noteworthy that the CNS function of MPS VII mice, transplanted after a myeloablative irradiation and evaluated with two behavioral tests, was not ameliorated in transplanted mice²⁶. Similarly, in a mouse model of MPS IIIA, neonatal BMT did not affect neuropathological storage²⁷. Likewise, BMT of MPS IIIB mice performed at 2-4 days of age after irradiation did not show any evident improvement in the brain²⁸. The authors concluded that BMT does not correct the CNS abnormalities of transplanted mice, even though the lack of improvement could be also attributed to the transplant procedure, in particular to radiation-induced toxicity in CNS or to the low engraftment of donor cells.

With the aim of evaluating the effect at the skeletal level, neonatal BMT has recently been tested in a MPS I mouse model²⁹. This study adopted a busulfan-based conditioning followed by a syngeneic BMT in the first days of life. Busulfan is a standard chemotherapy agent used in patients in combination with other drugs as a conditioning prior to HSCT, especially in leukemia, lymphoma, myeloproliferative disorders and MPSs. The use of busulfan in a neonatal experimental setting is an element of interest since it allows a better engraftment of donor cells in the brain compared with irradiation, which could also provide an effect on the neurological manifestations of the disease³⁰. Furthermore, by using this experimental regimen with busulfan in MPS I mice²⁹, the extent of engraftment obtained was higher, and the successive clinical improvement was very encouraging. The replacement of the hematopoiesis resulted in an increase in alpha-L-iduronidase (IDUA) activity in peripheral organs and, consequently, clearance of GAGs in plasma and various tissues. At 37 weeks of age, the reconstitution of normal hematopoiesis in MPS I mice was associated with a consistent amelioration of skeletal dysplasia. Radiographic analysis showed that the widths of the skull, of the zygomatic arches, and of the long bones in MPS I early treated mice were almost normalized. The bone morphometric parameters calculated by micro-CT revealed a 40-80% improvement in neonatally transplanted compared with untransplanted MPS I mice, approaching the values observed in WT. The authors also noticed a reduction of both hyperosteocytosis and lysosomal vacuolization in femur sections of neonatally transplanted MPS I mice. Overall, the magnitude of improvements correlated with the extent of hematopoietic engraftment, interestingly suggesting that the early

restoration of normal hematopoiesis provides a favorable impact on the bone development in MPS I. Therefore, BMT at a very early stage in life markedly reduces signs and symptoms of MPSs in animal models by preventing their development.

NEONATAL GENE THERAPY

Gene therapy also offers a potential therapeutic opportunity for MPSs. In theory, gene therapy for these disorders should act by providing the affected cells with enough enzyme. The mechanisms by which gene therapy could be effective include 1) the delivery of the gene directly to the cells mainly involved in these diseases and 2) the uptake by these cells of the missing enzyme secreted from other transduced cells acting as an enzyme source. Early gene transfer in the neonatal period overcomes some issues, which can occur in gene therapy performed in adulthood. First, in mature organisms affected by MPSs, the genetic defect has already caused irreversible pathological lesions mainly in bone and brain. Therefore, for MPSs and many other genetic diseases, gene therapy at birth will have a striking effect to arrest disease progression. In addition, if gene therapy is administered in adulthood, an immune response may rapidly eliminate transgenic protein expression precluding any favorable effect. In contrast, with an early intervention, the possibility to develop a vigorous immune system response toward the transgenic protein is less likely than in adulthood. Furthermore, in the case of an approach based on the use of genetically corrected hematopoietic stem cells, the autologous setting reduces the risks related to an allogeneic transplant (graft versus host disease,

GVHD) and provides potential advantage to patients lacking an HLA-matched donor.

In addition, a study in the MPS VII mouse model has shown that, as the disease progresses, more genes present altered expression, and this may account for the complex clinical phenotype. Only some of those changes in gene expression normalize when the treatment is initiated in animals with established disease, and this observation further supports the need of an early intervention³¹.

The two primary categories of somatic gene therapy consist in (1) the *in vivo* infusion of viral vector particles with the aim of transferring normal cDNA to the affected cells enabling them to express the missing protein or (2) the *ex vivo* transduction of patient's cells which could be subsequently infused (*Figure 1*). The first form of gene therapy is called *in vivo* because the gene vector is transduced to cells inside the patient's body. In the *ex vivo* procedure, cells from the patient's blood or bone marrow are cultured in the laboratory, exposed to the viral vector that is carrying the desired gene and then returned to the patient.

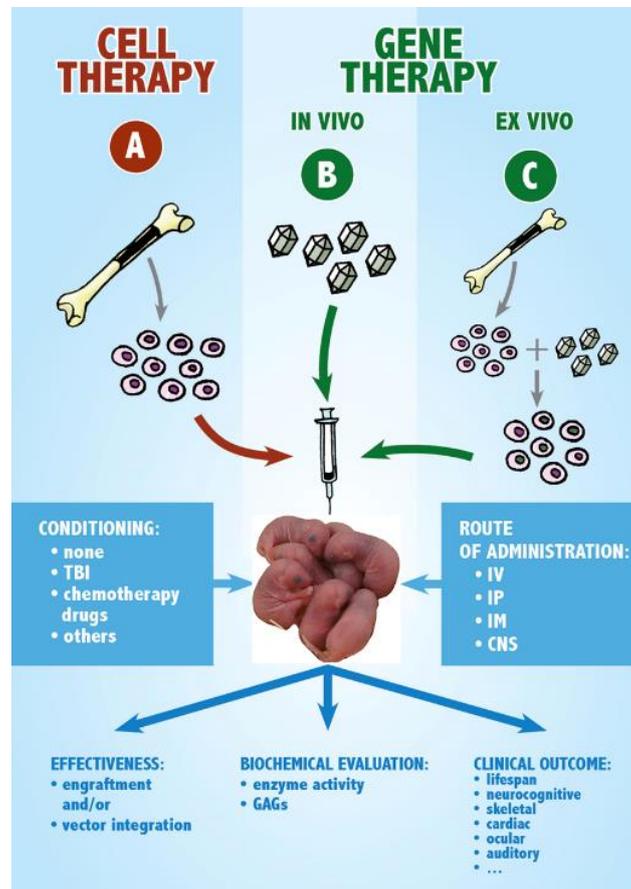


Figure 1. Scheme of the approaches employed for the neonatal therapy of MPS animal models. A) Cell therapy. Cells from the bone marrow (or alternative stem cell sources) of a healthy donor are collected and then transplanted into the affected newborn. B) Gene therapy in vivo. A viral vector carrying a functional copy of the defective gene is injected into the organism of the affected neonate. C) Gene therapy ex vivo. Bone marrow stem cells (or stem cells from other sources) are transduced ex vivo with a viral vector carrying a copy of the defective gene and then gene-corrected cells are transplanted into the affected neonate.

Two important factors in the experimental setting are the conditioning and the route of administration. The effectiveness of the approach is evaluated, and the outcome is observed focusing on biochemical and clinical parameters. Abbreviations: TBI, total body irradiation; IV, intravenous; IP, intraperitoneal; IM, intramuscular; CNS, central nervous system (route: intraventricular or intratechal); GAGs, glycosaminoglycans.

***In vivo* neonatal gene therapy**

In the last two decades, *in vivo* gene therapy studies in neonatal MPS mice and large animal models have been reported by using retroviral, adenoviral, lentiviral, and adeno-associated virus (AAV)-based vectors (*Table 1*). Systemic gene therapy could program some cells to secrete the lacking enzyme, which could be taken up by other affected cells via the mannose 6-phosphate receptor. The first report demonstrated the several advantages of an early *in vivo* gene therapy approach, and showed that intravenous AAV-mediated gene transfer in neonatal MPS VII mice may offer an efficient system to widespread correction³². Also in a CNS-directed gene therapy approach, the injection of recombinant AAV encoding human GUSB into both the anterior cortex than the hippocampus of newborn MPS VII mice has positive effect not only on the brain histopathology, but also improves cognitive function³³. Subsequently, Hartung et al. treated MPS I mice at birth with an AAV vector carrying the human IDUA cDNA and showed that AAV-IDUA gene transfer into newborn MPS I mice led to high levels of plasma and tissue enzyme activities, which were sufficient to normalize urine GAG levels and reduce lysosomal storage in a number of the main organs of treated mice³⁴. The effect of IDUA restoration on craniofacial and CNS parameters demonstrated significant improvements on these critical features of MPS I. Similarly, the *in vivo* injection of high doses of retroviral vector expressing IDUA resulted in the complete correction of biochemical and pathological evidence of disease in internal organs, bone, and brain^{35,36}. In particular, Liu et al. showed that MPS I mice that received high-dose of retroviral vectors had normal

echocardiograms, bone mineral density, auditory-evoked brain-stem responses, and electroretinograms³⁵.

With the advent of lentivirus-based vector technology, Kobayashi et al. estimated the possibility to perform gene therapy for MPS I by direct *in vivo* injection of a lentiviral vector³⁷. They compared the efficacy between newborn and young adult MPS I mice of lentiviral vector-mediated gene therapy, demonstrating a significantly greater advantage for mice treated neonatally. In particular, they showed that the neonatal administration of the lentiviral vector by a single intravenous injection leads to a sustained expression of active IDUA enzyme in multiple organs, including the brain, in which vector administration resulted in transduction of neurons in the brain. Interestingly, the authors were able to show a clear advantage in mice treated as neonates compared to those treated as young adults. Indeed, the disease manifestations were only moderately improved in this latter group, but almost normalized in mice treated earlier in life. When the vector was administered at birth, the IDUA activity resulted in decreased GAGs storage, prevention of skeletal abnormalities, a more normal gross appearance, and improved survival. The extent of transduction was dose-dependent, with the liver receiving the higher level of the vector, but other somatic organs reaching almost similar levels.

All these works provided different confirmations of the effectiveness of an early gene therapy approach on MPS mouse models, independently of the type of vector used.

In the last years accumulating evidence has provided support for the hypothesis that another critical point, which can impact the efficacy of

the neonatal gene therapy approach, is dependent not only on the virus type used but also on the route of administration.

The intracranial injection alone of an adeno-associated viral (AAV) vector in the mouse model of MPS IIIB resulted in improvement in lifespan, motor function, hearing, time to activity onset, and daytime activity level, but no effects on the lysosomal storage. A more recent report has described a novel approach using the combination of an intracranial injection of an AAV-based vector and an intravenous treatment with a lentiviral vector, showing better clinical, histological and biochemical features³⁸.

Although the majority of gene transfer experiments for the treatment of inherited or acquired diseases have mainly been performed in mice, large animal models clearly represent an important step in the preclinical evaluation of a gene therapy approach, as their responses are probably more predictive of the results in humans. Large animals are more similar in size to a neonate offering a more sophisticated disease modeling resembling several human features.

The efficacy of neonatal gene therapy has also been tested in large MPS animal models. The first successful application of neonatal gene therapy in large animals has been described by Ponder et al., who reported the clinical improvements seen in MPS VII dogs treated with a cGUSB-expressing retroviral vector as neonates³⁹. In particular, little or no corneal clouding and no mitral valve thickening have been observed. Radiographically, treated dogs had fewer skeletal abnormalities, and, therefore, they could run at all planned times of evaluation. Estimating the long-term effect of this treatment on MPS VII dogs, the authors concluded that neonatal gene therapy was able to

appreciably, even if not entirely, reduce bone and joint disease⁴⁰. It is notable that neonatal gene therapy in MPS VII dogs was not still effective in preventing lumbar spine disease⁴¹. Similarly, in newborn MPS VI cats treated with a feline N-acetylgalactosamine 4-sulfatase-expressing retroviral vector, the results indicated, at the bone level, improvements in some aspects such as femur length, articular cartilage erosion, mobility, but not a significant effect on cervical vertebral bone length⁴². Thus, the impact of neonatal gene therapy is different in different bones. It is of great interest to understand which bone is severely affected, when each bone starts to be affected by the disease or what difference is present in penetration of gene vector and its expression level in each bone.

A pivotal information can be evinced from the studies conducted in MPS I dogs⁴³. In this work, MPS I dogs were treated at birth with a gamma retroviral vector expressing the canine IDUA, and yielded a clinical effect derived from a stably expressed and circulating enzyme without showing an immune response⁴³. The authors speculated that the immaturity of the newborn immune system or the tolerance to canine IDUA epitopes could have contributed to prevent the production of anti-canine IDUA antibodies.

These findings on neonatal *in vivo* gene therapy are promising and pave the way for upcoming clinical trials, even if future studies in patients need always to assess the risks and benefits of the adopted vector.

***Ex vivo* neonatal gene therapy**

Ex vivo gene therapy consists of two steps: 1) infecting somatic cells *in vitro* by viral vectors, then 2) injecting gene-corrected cells *in vivo* into a newborn recipient organism. Such an approach is of particular interest in the case of MPSs: in fact, it allows the transduction of the defective gene directly into recipient cells, and the subsequent transplant of transduced cells in an autologous setting, avoiding the common immune issues of allogeneic transplantation. Moreover, the viral infection often allows reaching supraphysiological levels of protein expression in corrected cells, which could never be achieved using normal donor cells as a source of enzyme. This is another apparent advantage of *ex vivo* gene therapy, since it has been shown in animal models and in clinic that the extent of phenotypic correction is strongly related to the levels of enzyme activity reached in the recipient organism (organs or biological fluids) as a result of the treatment^{16,44}. In MPS animal models, this gene-correction strategy has mainly been performed on long-term hematopoietic repopulating cells, usually derived from bone marrow, which are cultured and transduced, and then transplanted into recipients with a standard procedure of bone marrow transplantation⁴⁴⁻⁴⁷.

Only a few papers related to neonatal *ex vivo* gene therapy in MPS animals have been published (*Table 1*). Simonaro *et al.* described the transplantation of retrovirally transduced bone marrow (BM) or newborn blood cells in MPS VI cats, some of which were very early in life. Cells transduced with the vector carrying the cDNA for the human arylsulfatase B, the enzyme deficient in MPS VI, were successfully engrafted and persisted for a long term in the cats, while the level of

enzyme activity reached was low, not sufficient to appreciate any clinical improvement. Notably, the authors employed as cell source not only BM, but also newborn blood, in an attempt to verify the feasibility of transplanting gene-corrected cord blood⁴⁸.

Other cell types have also been employed for gene correction. In 2003, Meng *et al.* focused on the CNS involvement in MPSs. They transduced human neural stem cells (hNSCs) with a retroviral vector, in order to express the human enzyme β -glucuronidase at supranormal levels; then, they injected corrected cells into the cerebral ventricles of immunodeficient MPS VII newborn mice. They identified the presence of hNSCs in host brains, the presence of β -glucuronidase activity, and a reduction of lysosomal storage. Unfortunately, these effects lasted only for a short time after transplantation because human cells rapidly underwent apoptosis⁴⁹.

Meyerrose *et al.* obtained even more encouraging results. They transduced human BM-derived mesenchymal stem cells (hBM-MSCs) by a lentiviral vector, forcing them to overexpress human β -glucuronidase. The intraperitoneal transplantation of corrected hBM-MSCs into neonatal NOD-SCID MPS VII mice leads to the engraftment of these cells in several organs and to their release of therapeutic levels of enzyme (nearly 40% of normal in serum), detected 2 and 4 months after the transplant. As a result, the storage of GAGs and the secondary elevated activities of other lysosomal enzymes were normalized. Notably, the authors attested even a clinical amelioration since treated mice showed an improvement in retinal function⁵⁰.

The described approaches, with their interesting initial results, are significant proofs of principle for the application of *ex vivo* gene therapy in the very early treatment of MPSs.

ADVENT OF NEWBORN SCREENING PROGRAMS AND FUTURE NEONATAL THERAPIES

Newborn screening on MPSs

It is inevitable to establish newborn screening systems for MPS patients to allow an early diagnosis and early therapy. There are two principal methods that are being developed and pilot studies are currently underway; one is the assay which measures the deficient enzyme in each MPS disorder, and the other one is the assay which measures primary storage substrates, GAGs. Both methods will analyze newborn dried blood spots (DBSs).

Enzyme assay method can be a useful tool in newborn screening for MPSs, measuring the enzyme activity of each deficient enzyme directly. This method is highly sensitive and specific. There are several reports of enzyme assays in MPS I^{51,52} and MPS II⁵³ patients. This group and others have developed direct multiple assays of enzyme activity in DBS samples by using tandem mass spectrometry (MS/MS) for newborn screening of lysosomal storage diseases^{54,55}. The enzyme assay can provide a diagnosis of the disease directly while the disadvantage is that the method cannot differentiate the pseudodeficiency from true positive patients. The micro-fluidics methodology is also being investigated for screening DBSs and has already applied in a full-population pilot study in Missouri⁵⁶.

Another method is to measure primary storage materials, GAGs. Several groups have developed highly sensitive, specific and inexpensive assay methods to distinguish patients with MPSs from healthy controls by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) systems. Tomatsu *et al.* measured heparan sulfate and dermatan sulfate levels in DBS samples from 6 neonatal MPS patients (4 MPS I, 1 MPS II and 1 MPS VII) and compared each GAG level from these MPS samples with that from healthy control samples (n = 326) in a double blind method. Both levels were markedly elevated in all six samples of MPS patients compared with the levels of control samples⁵⁷⁻⁶⁰. This group also measured 12 newborn samples with MPSs (6 MPS I, 1 MPS II and 5 MPS III) using both LC-MS/MS and high-throughput mass spectrometry (HT-MS/MS)⁶¹. The disaccharide levels of Δ DiHS-OS and Δ DiHS-NS from DBS samples with MPS I or MPS III were markedly elevated compared with those from control newborn samples (n = 22). Also in the case of MPS II samples, these levels were respectively 3 and 1.5 times higher than in controls⁶¹. De Ruijter *et al.* have also reported that the disaccharide levels of heparan sulfate and dermatan sulfate from newborn DBS samples obtained from MPS patients (11 MPS I, 1 MPS II and 6 MPS III) were significantly increased in all patients samples compared with controls⁶².

Therefore, these methods of newborn screening for MPS patients can be useful tools to make an early diagnosis for at least MPS I, II, and III. This group is starting a pilot study to measure specific GAGs from total 200,000 newborn DBS samples and to evaluate the new assay systems for MPSs newborn screening. This method provides a suggestion of a

high-risk group for MPSs and can be useful for assessing the clinical severity of the disease and monitoring the therapeutic efficacy and/or pharmacokinetics of the drug, while the second screening with enzyme assay is required for certain diagnosis.

Both methods have some limitations, such as false-positive/negative and costs in GAG assay and enzyme assay method. These issues still need to be resolved before establishing a newborn screening system for MPSs in the clinical practice.

Future neonatal therapies

As previously mentioned, ERT is a standard therapy for MPSs and is approved or under clinical trials in many countries for MPS I, MPS II, MPS IVA, MPS VI, and MPS VII patients. Patients treated with ERT showed clinical improvement of somatic manifestations and an enhanced quality of life. However, there are several limitations: 1) ERT is least efficacious on CNS and skeletal dysplasia, 2) the enzyme has a short half-life and high clearance from the circulation, 3) continuous ERT causes immunological problems, and 4) it is very expensive.

HSCT for MPS patients has been conducted prior to ERT; however, initial attempts of HSCT were controversial because of a high mortality rate. Several results on MPS animal models suggest that skeletal deformities and impaired growth development in MPS patients should be improved if HSCT is performed at earlier stages. HSCT has a risk for the development of mortality by GVHD, infections and additional complications. It is known that the severity of GVHD is influenced by the donor match and by pre-HSCT serotherapy. Anyway, conditioning regimens for HSCT have been markedly improved in each medical

facility, and well-trained staffs contribute to the least mortality of HSCT.

Busulfan is a standard chemotherapy drug usually given as a conditioning agent prior to HSCT. However, this drug may still induce severe side effects such as toxicity in lung and liver. Treosulfan (treo) is another alkylating cytotoxic agent with a supposedly less severe toxicity profile. It is most commonly used in the treatment of ovarian cancer. It is also increasingly used in HSCT, predominantly in non-malignant diseases. In European countries, treosulfan is approved and used efficiently and safely in pediatric patients before HSCT⁶³⁻⁶⁷. Pediatric MPS patients, who received a conditioning regimen consisting of treosulfan and others, achieved stable hematopoietic engraftment and stable donor chimerism without GVHD. The regimen with treosulfan could be an additional option when unrelated donor HSCT is considered for a patient with MPS⁶⁸ although the donor cell engraftment into the brain might be limited with this type of transplant. The long-term observation of HSCT with treosulfan is required. Neonatal or early HSCT can be more widely spread as main therapy for patients with MPS if treosulfan regimen is established in each type of MPS.

It should be noted that, to date, cord blood is a clinically useful source of HSCT for Hurler syndrome. Full-donor chimerism and normal enzyme levels are frequently achieved during the follow-up period^{15,69}. Cord blood transplantation also improves neurocognitive development in children with Hurler syndrome⁶⁹. In general, HSCT with cord blood has many advantages such as 1) easy procurement, 2) no risk to donors, 3) low risk of transmitting infections, 4) immune tolerance allowing

successful transplantation despite HLA disparity, and 5) immediate availability^{70,71}. The latter is clearly suitable for performing an early therapy. It is still critical to assess whether HSCT with cord blood might provide significant GVHD or not with more cases.

For what concerns neonatal gene therapy, animal studies for MPSs suggest that the viral and non-viral vectors have stably overexpressed within a long period of 10 years. In *ex vivo* gene therapy, retroviral vectors improved CNS disease in MPS I⁴⁵ and MPS IIIB⁷² mice. Direct infusion of viral vectors into the brain also improved CNS disease by *in vivo* gene therapy⁷³⁻⁷⁷. However, the efficacy of gene therapy for bone lesions remains unsolved. Several clinical trials in gene therapies for MPSs are currently underway in the United States. Phases I and II clinical studies for the therapy of Sanfilippo A syndrome using adeno-associated viral vector serotype rh. 10 carrying the human N-sulfoglycosamine sulfohydrolase (SGSH) and sulfatase-modifying factor (SUMF1) cDNAs are still ongoing⁷⁸.

The future ideal gene therapy practice is that the defective gene is replaced with a normal sequence at its natural location. This is advantageous compared with a virally delivered gene which includes the full coding and regulatory sequences when only a small proportion of the gene required to be changed, like a point mutation or small deletion and insertion. The expression of the partially replaced gene can be more consistent with normal cell physiology than the full gene accommodated by the viral vector.

Gene editing, or gene editing with engineered nucleases, is a type of genetic engineering representing an innovative technology. Through this system, a DNA fragment of interest is inserted, replaced, or

removed from a genome by using artificially engineered nucleases or "molecular scissors". The nucleases generate specific double-strand breaks (DSB) at preferred sites in the genome and employ the endogenous mechanisms of homologous recombination and non-homologous end-joining to restore the induced break point. At present, four families of engineered nucleases are in use experimentally or in clinical trials: Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), the CRISPR/Cas system (CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Cas: CRISPR-associated genes), and engineered meganuclease and re-engineered homing endonucleases⁷⁹⁻⁸¹ (Figure 2).

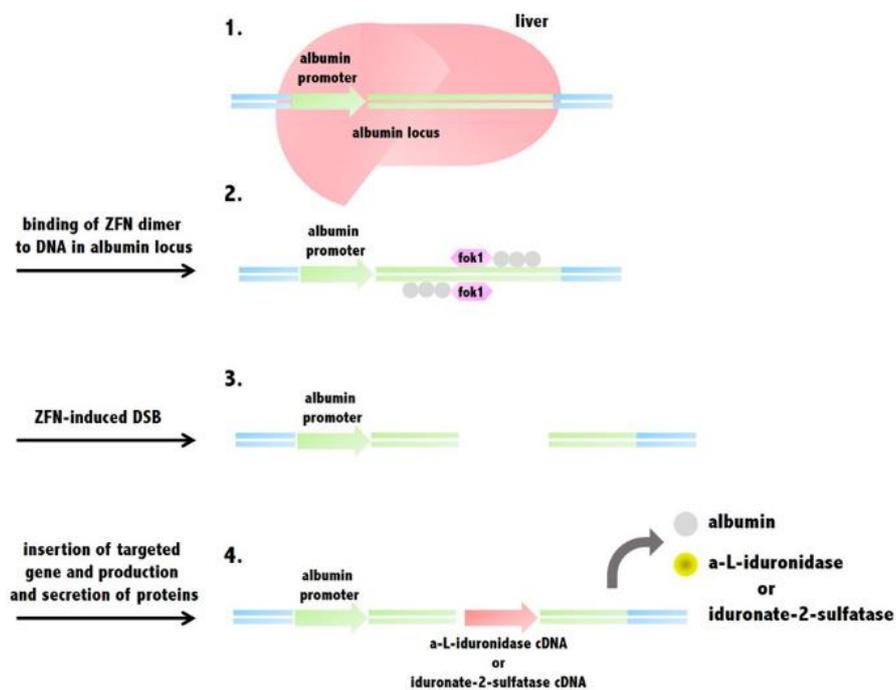


Figure 2. Gene Editing-based therapy in human albumin locus. 1. Gene Editing-based therapy for MPS I or II patients targets albumin locus sites in the liver. 2. ZFN binds to DNA sequences in the albumin locus and their *fokI*

nuclease domains dimerize between the binding sites. 3. ZFN induces DSB at the albumin locus in the genome of hepatocytes. 4. Alpha-L-iduronidase or iduronate-2-sulfatase genes are inserted into the albumin locus, and their proteins are continually produced from the liver into the blood. Abbreviations: ZFN, zinc finger nucleases; DSB, double-strand breaks.

For example, ZFN-induced targeting attacks defective genes at their endogenous chromosomal locations. Treatment of X-linked severe combined immunodeficiency has been administered by *ex vivo* gene correction with DNA carrying the IL-2 receptor common γ chain with the correct sequence⁸². However, one of the concerns about this new technology is that ZFNs may induce off-target mutations, apart from viral transductions. Many measures are under development to improve off-target detection and ensure safety prior to clinical use.

A particular use of gene editing is the *In Vivo* Protein Replacement Platform (IVPRP), which provides a broadly applicable genetic approach to enzyme replacement for LSDs⁸³. The IVPRP applies ZFN-mediated gene editing to insert precisely normal genes into the albumin locus of liver cells in patients (*Figure 2*). Successively, the normal enzyme is produced by the robust device that naturally drives albumin expression, leading to the production and secretion of the defective enzyme by the liver. At this moment, Hurler syndrome and Hunter syndrome are considered as candidate diseases, and the aim is to use this IVPRP approach to facilitate the liver to produce in patients therapeutic quantities of the normal enzymes, α -L-iduronidase and iduronate-2-sulfatase, respectively.

Non-viral vector systems such as *Sleeping Beauty* transposon or phiC31 recombinase-derived vector represent other approaches for safe gene transfer in MPSs^{84,85}. However, the IDUA gene expression obtained

with these methods decreases over time due to the immune response and the metilation of the CAG promoter.

Further long-term clinical studies are needed to evaluate the therapeutic efficacy of gene therapy for MPS patients.

It is also important to take into account that the clinical efficacy of any innovative cellular and gene therapy procedure is strictly dependent on providing the missing protein at a very early stage, before symptoms become apparent. In future, ERT should be envisaged in combined therapy with neonatal HSCT or gene therapy for MPS patients soon after the neonatal diagnosis (*Figure 3*). In fact, the combination of neonatal ERT and delayed (5 weeks of age) BMT has already been performed in a murine model of MPS VII with promising results⁸⁶.

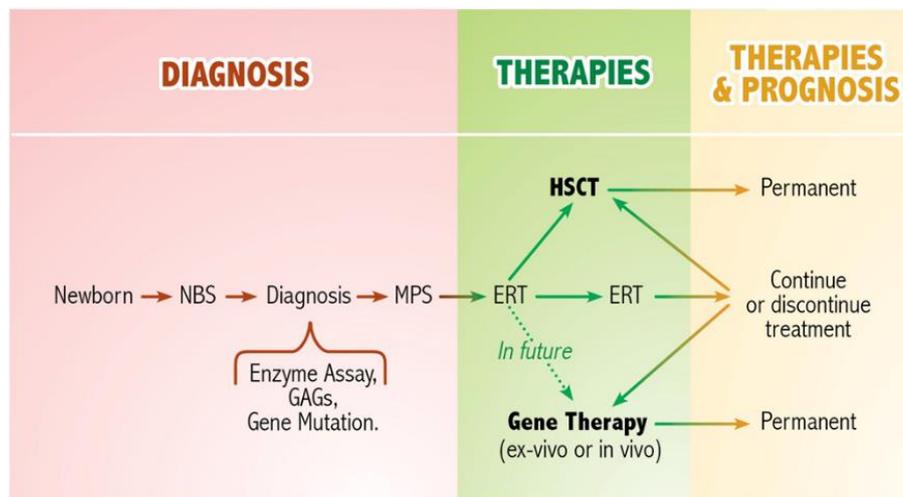


Figure 3. Potential scheme of future neonatal therapy for Mucopolysaccharidoses. Currently, ERT or HSCT are established treatments and have a beneficial effect on patients with MPSs. In future, neonatal cellular and gene therapy approaches combined with ERT could be applied to affected children diagnosed at birth through the newborn screening. Abbreviations: NBS, newborn screening; GAGs, glycosaminoglycans; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation.

To conclude, the perspective in the cure of MPSs should be more focused not only on the use of combined treatments, but also on the timing at which the therapies are given to patients, considering that the clinical outcome could be more favorable if the treatments are used to prevent instead of correcting the disease manifestations.

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CHAPTER 3

Neonatal umbilical cord blood transplantation halts skeletal disease progression in the murine model of MPS-I

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ABSTRACT

Umbilical cord blood (UCB) is a promising source of stem cells to use in early hematopoietic stem cell transplantation (HSCT) approaches for several genetic diseases that can be diagnosed at birth. Mucopolysaccharidosis type I (MPS-I) is a progressive multi-system disorder caused by deficiency of lysosomal enzyme α -L-iduronidase, and patients treated with allogeneic HSCT at the onset have improved outcome, suggesting to administer such therapy as early as possible. Given that the best characterized MPS-I murine model is an immunocompetent mouse, we here developed a transplantation system based on murine UCB. With the final aim of testing the therapeutic efficacy of UCB in MPS-I mice transplanted at birth, we first defined the features of murine UCB cells and demonstrated that they are capable of multi-lineage hematopoietic repopulation of myeloablated adult mice similarly to bone marrow cells. We then assessed the effectiveness of murine UCB cells transplantation in busulfan-conditioned newborn MPS-I mice. Twenty weeks after treatment, iduronidase activity was increased in visceral organs of MPS-I animals, glycosaminoglycans storage was reduced, and skeletal phenotype was ameliorated. This study explores a potential therapy for MPS-I at a very early stage in life and represents a novel model to test UCB-based transplantation approaches for various diseases.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) can cure or greatly ameliorate a wide variety of genetic diseases, including defects of hematopoietic cell production or function and metabolic diseases mainly affecting solid organs¹. In post-natal life, hematopoietic stem cells (HSCs) reside in the bone marrow (BM), so this was historically the first source of cells employed for HSCT. However, immediately after birth, HSCs can still be found in the fetal blood that flows in the umbilical cord vessels (umbilical cord blood, UCB). Unrelated donor UCB has several potential advantages over BM for HSCT, since it offers a relative ease of procurement, a greater degree of HLA (human leukocyte antigen)-mismatch, with increased probability to find a suitable donor and lower incidence of acute and chronic graft versus host disease (GVHD), and reduced risk of viral infections (like Epstein-Barr virus and Cytomegalovirus)¹⁻⁴. Furthermore, in the specific case of transplantation for inborn errors of metabolism (IEMs), UCB transplantation (UCBT) shows two significant extra-advantages over BM transplantation (BMT)^{3,5-7}. First, the availability of cells to transplant is more rapid, thanks to the augmented probability to find HLA-matched donors and the existence of cord blood banks where UCB units are stored frozen and ready to use. This factor is of primary importance because in many IEMs the timing of the treatment has a strong impact on patient outcome. Additionally, more patients transplanted with UCB achieve full donor chimerism and thus can obtain a normalization of the deficient enzyme levels in biological fluids, with consequent clinical benefits^{3,6,8}.

New strategies and novel developments are expected to improve engraftment and reconstitution, and to enable *in utero* or neonatal UCB-based transplantation for early therapy of these diseases^{2,9}. Thus, convenient small animal models of these disorders are essential to investigate these developing strategies in the field of HSCT, including the use of alternative cellular sources and/or genetically modified HSCs.

Even though immunodeficient mouse models of many genetic disorders are available, in which the transplantation of human HSCs is feasible, many diseases lack an immunocompromised model that could fully recapitulate their clinical manifestations. In the case of Mucopolysaccharidosis type I-Hurler syndrome (MPS-IH), a lysosomal storage disease due to mutations in the α -L-iduronidase (*IDUA*) gene, immunocompetent mouse models have been deeply characterized for several features typical of this complicated disorder¹⁰⁻¹³. Recently, MPS-I immunocompromised models have been generated, but some aspects representative of the disease are not completely investigated yet^{14,15}. In this disorder, the absence of *IDUA* activity causes the progressive accumulation of glycosaminoglycans (GAGs) in tissues, which leads to multiple organ dysfunction, with central nervous system involvement and various skeletal anomalies known overall as dysostosis multiplex^{16,17}. The current first line therapy for MPS-IH is HSCT, which provides a constant reservoir of enzyme replacement through the engraftment of donor cells, and the use of UCB as stem cell source seems to guarantee the best results. However, transplantation is not completely effective in ameliorating bone abnormalities and neurocognitive dysfunctions, especially when it is performed late in

childhood^{7,8,18,19}. Clinical and preclinical evidences attest that the precociousness of the treatment is critical to prevent long-term pathological consequences²⁰. For this reason, we tested an UCBT approach at early age in MPS-I murine model¹⁰, to investigate a novel and promising therapeutic strategy. Very few data are present in the literature about murine UCBCs and their transplantation. Attempts to mimic UCBT have been made with either fetal liver cells, blood or BM collected from mouse fetuses during the last third of pregnancy, or newborn blood²¹⁻²⁷, but, to our knowledge, no published data exist about the transplantation of murine UCB into newborn recipients, in particular in a mouse model of disease attesting a clinical correction. Building upon the data from our previous study where we observed that the transplantation of normal BM into newborn MPS-I mice, soon after the placental protection, can prevent GAGs accumulation in multiple organs and the distinctive skeletal dysplasia²⁸, in this study we provide an extensive description of murine UCB cells (UCBCs) features, in comparison with adult BM cells (BMCs). We characterized UCBCs *in vitro*, by flow cytometry and colony forming cell (CFC)-assay, and assessed the repopulating ability of UCBCs in conditioned adult and newborn wild-type (WT) mice. Finally, we focused on the pathological setting and investigated a novel treatment strategy based on the transplantation of UCBCs in MPS-I mice at birth. We extensively evaluated the outcome of this therapy, regarding restoration of enzyme activity, reduction of GAG deposits in plasma and visceral organs, and correction of the skeletal phenotype.

RESULTS

Collection of UCBCs and their comparison with adult BMCs *in vitro*

We collected UCBCs at gestational day E18 from C57BL/6 pregnant dams, which carried a mean number of fetuses/dam of 6.97 (standard deviation [SD] 1.81; n=72 dams). The mean number of UCBCs collected from each dam was 1.38×10^6 (SD 4.51×10^5), and it varied proportionally with the number of fetuses/dam (data not shown). The mean number of cells obtained from each fetus was 19.8×10^4 (SD 5.64×10^4) (*Figure 1A*). Hematopoietic cells belonging to different lymphoid and myeloid lineages were found both in UCB and adult BM, as shown in the representative flow cytometry panels in *Figure 1B*. However, the proportion of lymphocytes (T cells and B cells) and of myeloid cells (monocytes/macrophages and granulocytes) was higher in BM than in UCB, suggesting that UCB could contain less mature cell populations (Supplementary Table S1). Interestingly, Ter119⁺ erythrocytes were very few in BM after lysis but remained at a high percentage in UCB, probably because they are mostly immature and resistant to hypotonic shock (*Supplementary Figure S2*). Regarding the HSCs subset easily detectable within adult BMCs by Lin⁻Sca-1⁺c-kit⁺ (LSK) staining, in UCBCs specimens there was a reduced proportion of LSK cells (*Figure 1B*). In the colony-forming cell (CFC) assay, performed to investigate the functionality of the hematopoietic progenitors, a similar frequency of colonies was found in UCB and BM (median 35.0 colonies/plate in UCB, range from 16 to 112, and 39.0 colonies/plate in BM, range from 18 to 78; p=1) (*Figure 1C*). However,

in UCB the majority of the colonies (93.2%) had a peculiar morphology, consisting of colonies containing large blast-like cells on a single layer (*Figure 1D-E*). These cells resemble the previously defined High Proliferative Potential-Colony-Forming Cells (HPP-CFC), primitive multipotent progenitor cells absent in BM-derived colonies²³. Excluding HPP-CFCs, the relative distribution of the other colony subtypes (CFU-GEMM, BFU-E, CFU-GM) did not differ between UCB and BM ($p=0.06$, Chi-square test) (*Figure 1D*). The different subtypes of UCB and BM hematopoietic colonies were morphologically indistinguishable (*Figure 1E*).

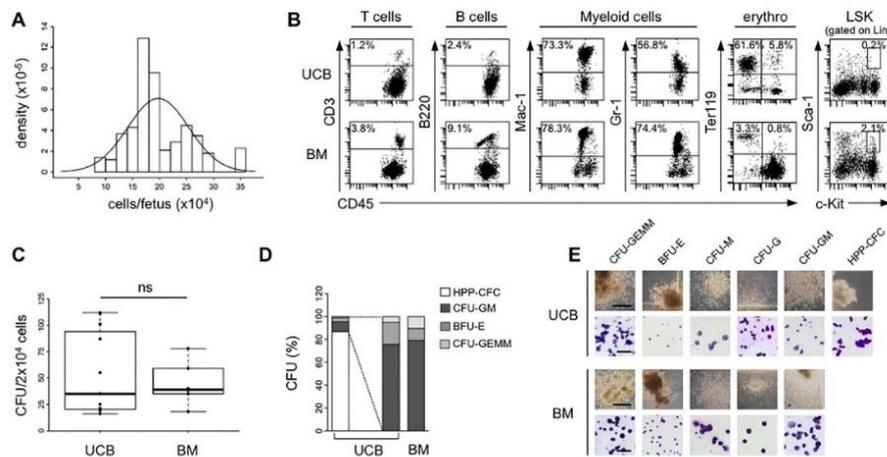


Figure 1. Murine UCBCs have unique features compared with BMCs.

A. Number of UCBCs obtained at day E18 from each fetus ($n=502$ fetuses). The distribution of the medium number of cells per fetus was represented by density histogram with Gaussian approximation. **B.** Representative flow cytometry analysis of UCB and BM hematopoietic subpopulations: T cells ($CD45^+CD3^+$), B cells ($CD45^+B220^+$), myeloid cells ($CD45^+Mac-1^+$ and $CD45^+Gr-1^+$), erythroid cells ($TER-119^+$), and LSK cells ($lin^-Sca-1^+c-Kit^+$). Percentages of lymphocytes and myeloid cells were referred to $CD45^+$ leukocytes, percentage of $Ter119^+$ was referred to all cells (after hypertonic treatment), and percentage of LSK cells was referred to Lin^- leukocytes. **C.**

Absolute number of hematopoietic colonies detected on methylcellulose at day 14 after plating 2×10^4 UCB or BM cells/petri (n=11 UCB, n=6 BM). Data are represented by boxplot graphs, showing the exact data values by black dots. P=1 with 2-sided Wilcoxon unpaired test. D. Barplot with percentage of the different subtypes of HPP-CFC, CFU-GEMM, BFU-E and CFU-GM (CFU-M, CFU-G, and CFU-GM) among the total number of colonies obtained from UCB or BM. E. Representative photographs of the different subtypes of hematopoietic colonies in UCB and BM (10X magnification, bar: 400 μ m) and of their cytopsin preparations stained with May-Grumwald Giemsa (200X magnification, bar: 200 μ m). CFU-GEMM = Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte; BFU-E = Burst-Forming Unit-Erythroid; CFU-M = Colony-Forming Unit-Macrophage; CFU-G = Colony-Forming Unit-Granulocyte; CFU-GM = Colony-Forming Unit-Granulocyte, Macrophage; HPP-CFC = High Proliferative Potential-Colony-Forming Cell.

UCB contains long-term multi-lineage repopulating hematopoietic stem cells

Before performing the transplantation of UCBCs into newborn MPS-I mice, we assessed whether they were able to rescue lethally-conditioned adult mice, to differentiate into cells of lymphoid and myeloid lineages, to persist long-term, and to repopulate in serial transplants. In an initial set of experiments, lethally-irradiated adult C57BL/6-CD45.1 mice were transplanted with 5×10^5 CD45.2⁺ UCBCs (adult UCBT group, aUCBT) or with the same number of murine adult BMCs (adult BMT group, aBMT). At 1 month after transplantation, the short-term engraftment of donor cells was assayed in peripheral blood (PB), BM, spleen, and thymus of the recipients, by flow cytometric analysis of the leukocytes marked with anti-CD45.1 and anti-CD45.2 antibodies (*Figure 2A*). In the aUCBT group, median engraftment in PB was 71.7% (range from 66.9% to 74.3%), even higher than the one in the aBMT group that was 58.4% (range from 42.3% to 68.3%;

p=0.03). In the BM, the median donor chimerism was 82.5% in the aUCBT group and 83.3% in the aBMT group, while it was, respectively, 62.7% and 69.3% in the spleen, and 22.8% and 23.2% in the thymus, without significant differences between UCBCs and BMCs (p=1, p=0.90, p=0.56, respectively). We next assessed if it was possible to use lower doses of UCBCs (2.5×10^5 and 1×10^5 cells/mouse) to establish long-term, stable chimerism. Rates of engraftment (number of surviving mice with $\geq 1\%$ donor cells/total number of transplanted mice) were 100% for all the tested doses. While at 1 month after transplantation the level of donor cell engraftment in PB depended on the transplanted cell dose, beginning from 4 months after transplantation the engraftment reached values over 90% in all the experimental groups (*Figure 2B*). More importantly, UCBCs showed long-term repopulation ability, since PB engraftment was maintained up to 12 months after aUCBT. The engraftment in other hematopoietic organs was assayed at 4 months after transplantation, and it reached a median of 98.2% in BM, 93.5% in spleen, and 92.1% in thymus (data not shown). The presence of cells arisen from the original UCBCs (CD45.2⁺ cells) in both lymphoid (T and B cells) and myeloid (monocytes/macrophages and granulocytes) lineages was attested in BM, spleen and thymus by flow cytometry (*Figure 2C*). CD45.2⁺ LSK cells were also found in the BM of recipient mice, suggesting that, even if LSK cells were detected as a very rare population in UCB, UCBCs were able to repopulate also the HSCs pool in the recipients' BM (*Figure 2C*). To evaluate the functionality of UCB-derived hematopoietic progenitors, CD45.2⁺ cells were sorted 4 months after aUCBT from the BM of recipients and were tested in a CFC-assay,

showing the differentiation in colonies belonging to all the different subtypes (*Figure 2 D-E*). Finally, a secondary transplantation assay into lethally-irradiated CD45.1 recipients was performed, to verify whether UCBCs contained long-term HSCs. The presence of sustained and durable levels of PB engraftment in secondary mice confirmed self-renewal and long-term repopulation capability of UCB-derived HSCs (*Figure 2F*). CD45.2⁺ mature subpopulations and LSK cells were also present in BM of secondary mice (data not shown).

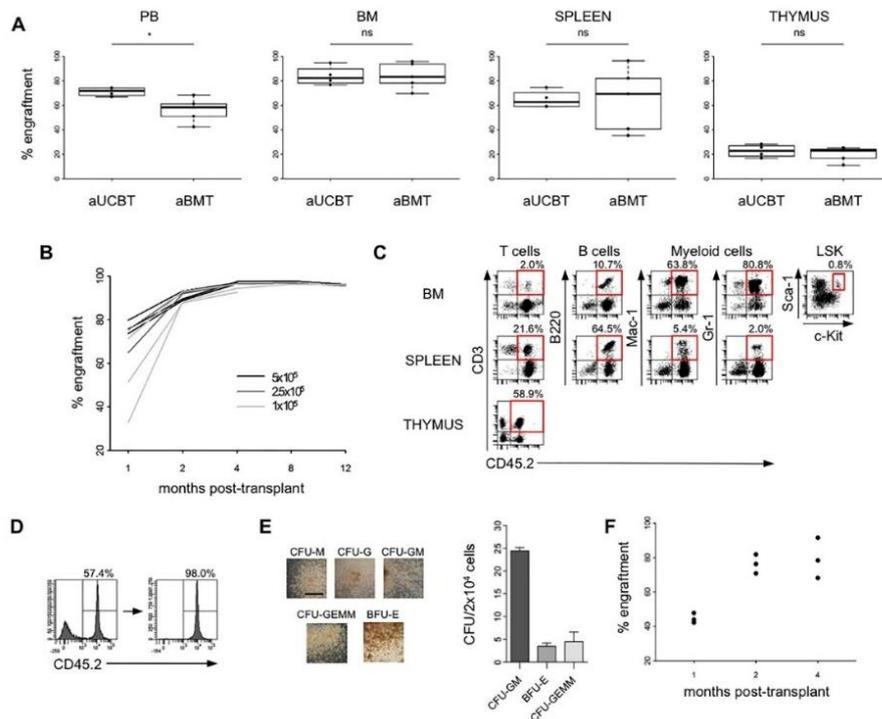


Figure 2. Murine UCBCs demonstrate long-term multi-lineage hematopoietic repopulating activity in adult transplantation setting.

A. Levels of donor chimerism [donor CD45 cells/(donor + host CD45 cells) x 100] were determined by flow cytometry in the hematopoietic organs of adult lethally-irradiated recipients at 1 month after the transplantation of 5×10^5 UCBCs (aUCBT) or BMCs (aBMT) ($n=4$ aUCBT, $n=5$ aBMT). * $p \leq 0.05$ by Wilcoxon test. **B.** Levels of chimerism analyzed serially in the PB of recipient

mice between 1 and 12 months after the transplantation of 5×10^5 , 2.5×10^5 , or 1×10^5 UCBCs/mouse (each line in the plot represents a single mouse). **C.** Representative lineage distribution of UCB-derived cells in the BM, spleen, and thymus of recipient mice at 4 months after aUCBT. Dot plots to determine donor-derived T cells ($CD45.2^+CD3^+$), B cells ($CD45.2^+B220^+$), myeloid cells ($CD45.2^+Mac-1^+$ and $CD45.2^+Gr-1^+$), and LSK cells ($CD45.2^+lineage^+Sca-1^+c-Kit^+$) are shown. **D.** FACS sorting of $CD45.2^+$ UCB-derived cells from the BM of a primary aUCBT recipient at 4 months after transplantation. **E.** Representative photographs and count of the different subtypes of hematopoietic colonies on methylcellulose formed by UCB-derived ($CD45.2^+$) BM sorted cells (10X magnification, bar: $400 \mu\text{m}$). **F.** Donor chimerism in the PB of secondary mice after the transplantation of 3×10^6 UCB-derived ($CD45.2^+$) BM sorted cells ($n=3$ recipient mice). Each black dot in the plot represent a single mouse, analyzed at 1, 2, and 4 months after transplant.

Transplantation of UCBCs in the neonatal setting

For the transplantation of newborn mice, we adopted a previously established protocol with few modifications²⁸. After conditioning with busulfan, $CD45.2$ newborn mice were intravenously transplanted with either $CD45.1^+$ UCBCs (neonatal UCBT group, nUCBT) or adult BMCs (neonatal BMT group, nBMT). The cell dose we defined to transplant (2×10^5 cells/mouse) was ideally comparable with the mean number of UCBCs harvested from a single fetus. At 1 month after transplantation, no difference in the PB engraftment was observed between nUCBT group (median: 18.5%, range from 1.0% to 71.8%) and nBMT group (median: 30.5%, range from 2.6% to 66.1%; $p=0.10$, including in our analysis only successfully transplanted mice with donor chimerism $\geq 1\%$) (Figure 3A). nUCBT mice with PB engraftment $\geq 50\%$ at 1 month were analyzed serially at 2 and 4 months, and the levels of engraftment in PB increased over time, approaching full donor chimerism (Figure 3B). The engraftment in BM, spleen, and thymus,

the differentiation of transplanted UCBCs in lymphoid and myeloid lineages, and the retention of LSK cells were evaluated at 6 months post transplantation, and the results were comparable with the ones obtained in aUCBT mice (*Supplementary Figure S1* and data not shown). Thus, we could also confirm in the neonatal setting the hematopoietic repopulation ability of UCBCs.

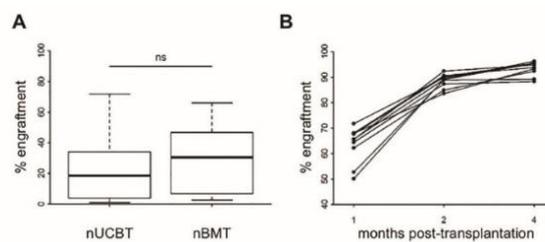


Figure 3. Murine UCBCs confirm long-term multi-lineage hematopoietic repopulating activity in neonatal transplantation setting.

A. Levels of donor chimerism were determined by flow cytometry in the PB of busulfan-conditioned newborn mice at 1 month following the transplantation of 2×10^5 UCBCs (nUCBT) or BMCs (nBMT) cells ($n=68$ nUCBT, $n=28$ nBMT; $p=0.10$ by Wilcoxon test). **B.** Serial analysis of donor chimerism in the PB of nUCBT recipient mice performed at 1, 2, and 4 months after transplant ($n=10$, each line in the graph represents a single mouse).

Engraftment and biochemical features of MPS-I mice receiving nUCBT

To investigate whether nUCBT could represent a curative treatment for metabolic diseases, we applied the settled protocol to the MPS-I mouse model. Newborn MPS-I and WT mice were transplanted with healthy UCBCs and evaluated at 20 weeks of age for their PB engraftment, IDUA activity in organs, GAGs accumulation in organs and plasma, and skeletal phenotype. PB engraftment did not differ between MPS-I and WT mice at the time of sacrifice (median 38.7% in MPS-I, range from 1.6% to 96.2%; 9.8% in WT, range from 1.0% to 94.8%; $p=0.24$)

(*Figure 4A*). Among MPS-I nUCBT mice, 5 of 12 mice presented a high hematopoietic chimerism, defined as more than 50% donor CD45.1⁺ cells in PB at 20 weeks after nUCBT (median engraftment: 93.3%, range from 55.7% to 96.2%). Hence, we included this subgroup of highly engrafted mice (named MPS-I nUCBT-hi) in all the studies reported hereafter. Five of 12 mice, instead, had a low hematopoietic chimerism, defined as less than 10% donor CD45.1⁺ cells in PB at 20 weeks after nUCBT, and were grouped as low engrafted mice (MPS-I nUCBT-lo). IDUA activity was evaluated in the spleen, liver, lung, kidney, and heart of MPS-I nUCBT mice compared with age-matched untreated WT and MPS-I mice. IDUA activity, which is absent in MPS-I mice and not restored in MPS-I nUCBT-lo mice, was partially increased in MPS-I nUCBT-hi mice in all the tissues analyzed, particularly in spleen, where the average values in MPS-I nUCBT-hi mice reached 40% of average WT values (*Figure 4B*). In the same harvested tissues, we quantified GAG levels, showing that MPS-I nUCBT-hi animals displayed a statistically significant reduction in GAGs storage material in all organs, in comparison with untreated MPS-I mice ($p \leq 0.03$ for all organs) (*Figure 4C*). The average reduction on MPS-I is over 50% for spleen, heart, lung and kidney. In particular, in spleen, liver, and lung of MPS-I nUCBT-hi animals GAG levels completely normalized (MPS-I nUCBT-hi vs. WT, $p=0.76$, $p=1$, and $p=0.11$, respectively) (*Figure 4C*). To further confirm the occurred correction, we measured the levels of plasma GAGs (HS-0S, HS-NS, DS, and mono-sulfated KS), showing that the levels of these GAGs were significantly reduced in MPS-I nUCBT-hi compared to untreated MPS-I mice (*Figure 4D*). Of note, in MPS-I nUCBT-hi mice the level

Figure 4. Neonatal UCBT prevents GAGs accumulation in MPS-I mice.

A. Donor chimerism (percentage of CD45.1⁺ cells) determined by flow cytometry in the PB of recipient MPS-I and WT mice at 20 weeks (time of sacrifice) after nUCBT (n=12 for MPS-I, n=12 for WT; p=0.24 by Wilcoxon test). Dashed line indicates the level of 50% donor engraftment, and identifies the highly-engrafted mice group (with ≥50% donor cells in PB, nUCBT-hi). **B.** IDUA activity in spleen, liver, lung, kidney, and heart of WT (n=8), MPS-I (n=8), MPS-I nUCBT-hi (n=5), and MPS-I nUCBT-lo mice (n=5). **C.** GAG levels in the indicated organs of the same WT, MPS-I, MPS-I nUCBT-hi, and MPS-I nUCBT-lo mice. **D.** Levels of ΔDiHS-0S, ΔDiHS-NS, ΔDi-4S, and mono-sulfated KS in the plasma of the mice. *p≤0.05, **p≤0.01, ***p≤0.001 by Wilcoxon test.

Prevention of dysostosis in MPS-I mice receiving nUCBT

Dysostosis multiplex is the well-known skeletal consequence of MPS-I in humans and mouse models. In particular, the MPS-I model adopted in this work shows abnormal craniofacial bone morphology and progressive thickening of the long bone segments. At the age of sacrifice (20 weeks) radiographic analyses confirmed a marked increase in the width of the skull and of the zygomatic arches in untreated MPS-I mice compared to WT animals (*Figure 5A*). Instead, in MPS-I nUCBT-hi mice, a significant reduction of these parameters was observed (skull width p=0.008; zygomatic arch width p=0.005, MPS-I nUCBT-hi vs. untreated MPS-I mice).

A similar trend was observed in the femur and humerus, where the thickening and the meta-diaphyseal sclerosis of the skeletal bones of MPS-I mice revealed by radiographic analysis were prevented in MPS-I nUCBT-hi animals (*Figure 5B*).

Considering that busulfan toxicity *per se* could cause a reduction in bone dimensions of treated mice regardless of their genotype, we adopted a regression model capable of separating the adverse effect of

busulfan treatment from the therapeutic effect of transplantation on MPS-I²⁸. By this analysis, we obtained the confirmation of the differential effect of the treatment on MPS-I attributable to transplantation only (Supplementary Table S2). Moreover, considering the MPS-I nUCBT-lo group, the improvement in radiographic measurements was limited compared to MPS-I nUCBT-hi, confirming the importance of high donor chimerism for disease correction (*Figure 5A and B*).

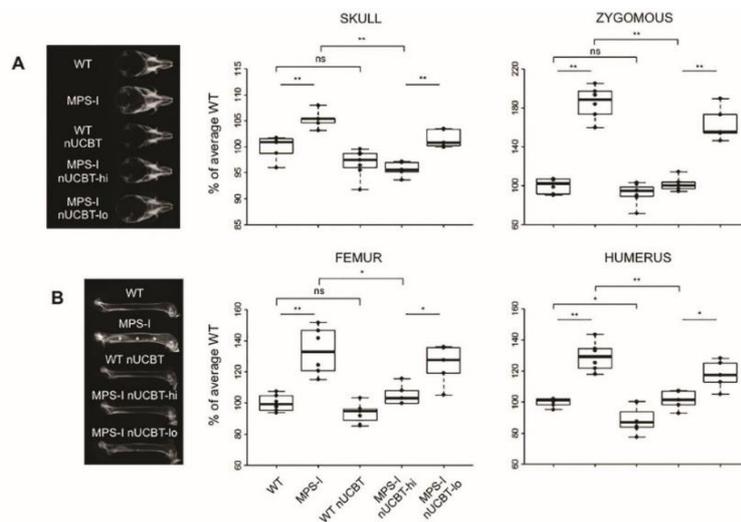


Figure 5. Neonatal UCBT prevents bone thickening in MPS-I mice.

A. On the left, representative radiographs of the skull of 20-weeks-old WT, MPS-I, WT nUCBT, MPS-I nUCBT-hi, and MPS-I nUCBT-lo mice. On the right, measurements of the skull width and zygomatic width, performed on radiographs of WT ($n=6$, 3 males and 3 females), MPS-I ($n=6$, 3 males and 3 females), WT nUCBT ($n=7$, 3 males and 4 females), MPS-I nUCBT-hi ($n=5$, 3 males and 2 females), and MPS-I nUCBT-lo mice ($n=5$, 3 males and 2 females). **B.** On the left, representative radiographs of the femur of 20-weeks-old WT, MPS-I, WT nUCBT, MPS-I nUCBT-hi, and MPS-I nUCBT-lo mice. The increase in meta-diaphyseal bone density observed in MPS-I (asterisks) is significantly prevented in MPS-I nUCBT-hi mice. On the right, measurements of the femur and humerus widths, performed on the radiographs of the same animals as in panel A. * $p \leq 0.05$, ** $p \leq 0.01$, by Wilcoxon test.

Micro-computed tomography (micro-CT) scans and histomorphometry performed on the femurs of male mice again highlighted the improvement of the skeletal phenotype in the MPS-I nUCBT-hi group. 2- and 3D micro-CT images revealed that the endocortical perimeter of MPS-I femurs appeared distinctly irregular at 20 weeks and returned to normal in MPS-I nUCBT-hi mice (*Figure 6A*). Specifically, all the examined parameters (total cortical area, cortical bone area, medullary area, and cortical thickness) were ameliorated in MPS-I nUCBT-hi mice, demonstrating the impact of the high donor engraftment on femoral architecture (*Figure 6B*). In addition, comparative histomorphometric analysis of the femur cortical thickness at mid-diaphysis and the area of the osteocytic lacunae confirmed the benefit of nUCBT on bone abnormalities in MPS-I mice (*Figure 6C* and data not shown).

Considering the impact that osteoclastogenesis seems to have on MPS-I disease²⁹, we determined the effect of nUCBT on osteoclast numbers and function. There were no significant differences in the ability of BM cells derived from untreated WT and MPS-I mice to differentiate into TRAP-positive multinucleated osteoclasts *ex vivo* and in their resorptive capacity *in vitro* when cultured on dentine slides (*Figure 6D*). Nonetheless, nUCBT treatment caused increased osteoclastogenesis regardless of the mice's genotype ($p \leq 0.05$, treated vs. untreated mice) (*Figure 6E*), although an effective reduction of bone mineral density could not be found *in vivo* (*Supplementary Figure S3*).

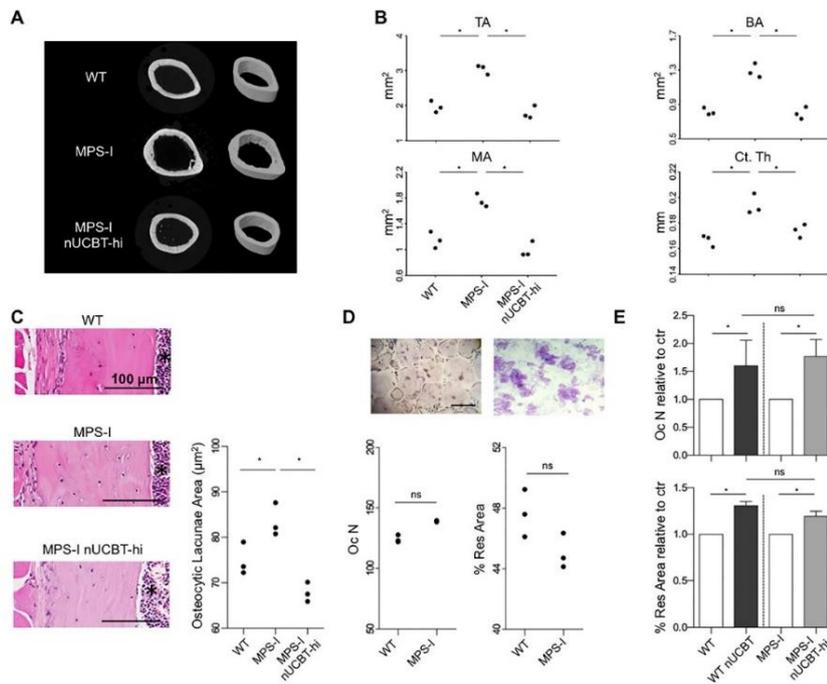


Figure 6. Neonatal UCBT improves cortical bone architecture in MPS-I mice.

A. Representative 2D and 3D micro-CT images showing regions of femoral cortical bone in WT, MPS-I, and MPS-I nUCBT-hi 20-weeks-old male mice.

B. Graphs representing the measurement of total area (TA/mm²), bone area (BA/mm²), medullary area (MA/mm²), and cortical thickness (Ct.Th/mm) of 3 mice per group (WT, MPS-I, and MPS-I nUCBT-hi).

C. Representative hematoxylin and eosin stained histological sections of the femur cortical bone at the mid-diaphysis are shown in the panels on the left. The graph illustrates the measurement (mean \pm SD) of the area of the osteocytic lacunae within the femur cortical bone of 3 mice per group (WT, MPS-I, and MPS-I nUCBT-hi). The BM cavity is indicated by an asterisk. Bar: 100 μ m.

D. Representative pictures of TRAP-positive multinucleated osteoclasts differentiated *ex vivo* (on the left, magnification 10X; bar: 300 μ m) and their resorption plots on dentin slices (on the right, magnification 20X). Quantification of number and resorptive capacity of osteoclasts obtained by *ex vivo* differentiation of BM cells arised from untreated WT and MPS-I mice (n=3 male mice per group).

E. Fold increase of the number and resorptive capacity of the osteoclasts obtained from treated mice, relative to control (untreated mice of the respective genotype) (mean \pm SD). * $p \leq 0.05$ by Wilcoxon test.

DISCUSSION

UCB is a clinically useful reservoir of HSCs and progenitor cells for the treatment of a wide variety of genetic diseases, particularly attractive for transplantation of infants and small children.

To fully realize the therapeutic potential of UCBT early after birth, it is fundamental to develop novel tools to test its efficacy in different defects. MPS-I offers an ideal model, since the relevance of UCBT in the treatment of this condition is well-known in clinic.

In this study, we demonstrate that the transplantation of murine UCBCs into lethally-irradiated congenic recipients long-term reconstitutes all blood cell lineages. Moreover, the BM of recipients contains cells capable of reconstituting the hematopoietic system of secondary hosts. Furthermore, in the neonatal setting, MPS-I mice transplanted with UCBCs show high levels of chimerism with the donor healthy cells, that are both well tolerated and therapeutic. Indeed, the long-term engraftment results in the partial restoration of IDUA enzyme activity, clearance of GAGs storage, and significant improvement in altered bone architecture, with prevention of the skeletal phenotype.

In contrast to adult murine BM, the features of murine UCB have been poorly investigated. In a few studies, blood from late fetal and newborn mice has been employed, due to the similar hallmarks with UCB obtained at birth in human beings. In the current study, we used UCB collected from murine fetuses at embryonic day 18. Even if UCB contained few nucleated cells, the collected cell population comprised the most representative committed lineages (T cells, B cells, and

myeloid cells), although in different proportions if compared to BMCs. Notably, the majority of T cells are immature, with a double positive CD4⁺CD8⁺ phenotype and low levels of TCR α/β , as similarly reported for human UCB^{27,30}. Both the low percentage of mature T cells and the weak reactivity of the numerous immature T cells can be responsible for the reduced incidence of GVHD in patients transplanted with UCB³¹. The almost complete absence of mature lymphocytes and the reduced number of innate immunity cells are allowed by the intra-uterine protection during fetal life³². Differently from murine adult BM, murine UCB is characterized by the presence of Ter119⁺ immature red blood cells resistant to hypotonic shock, including a population of nucleated red blood cells. These data are consistent with similar results reported for human UCB, which contains two distinct red cells populations, a minority of rapidly lysed cells and a majority of slowly disrupted cells³³.

Regarding the HSCs subset, the proportion of LSK cells, easily detectable within adult BM, was very low in UCB. This is consistent with the findings of Migishima et al., who stated that murine UCB virtually lacked cells with the LSK phenotype representative of adult BM-derived HSCs²⁴. Considering that UCB cells successfully reconstituted lethally irradiated recipients, the authors conclude that some phenotypic differences between BM and UCB HSCs may exist. A possibility is that UCB HSCs do not express Sca-1, since they found a population of Lin⁻c-Kit⁺ cells among the side population. Another possibility is that UCB HSCs express Mac-1 similarly to fetal liver HSCs, and consequently a LSK phenotype can be observed only if the anti-Mac-1 antibody is removed from the anti-lineage cocktail^{33,34}.

Furthermore, the majority of the colony progenitors was constituted by multipotent precursors that give rise to colonies with a peculiar blast-like morphology when cultured *in vitro*, resembling the previously-defined HPP-CFC²³. As already reported, this population of hematopoietic cells demonstrating HPP-CFC activity begins to be present in the yolk sac and in the embryo and represents the earliest multi-potential precursors within the hematopoietic hierarchy than can be cultured without stromal support³⁵. Similarly, human UCB cultures contain a higher proportion of immature, late developing, multi-potential colony-forming cells than adult BM cultures³⁶.

Even though these findings indicate that UCB has a different composition compared to BM, UCBCs can engraft with an extent similar to adult BM. In the congenic context, we do not observe any post-transplantation delay in hematopoietic recovery, differently than previously reported by Li *et al.* in an allogeneic UCBT model²⁶. Notably, the persistence of donor-derived lymphoid and myeloid lineages over 4 month after transplantation demonstrates the long-term function of the HSCs contained in UCB, considering that most precursors and short-term HSCs that repopulate soon after transplantation are short-lived and disappear within 3 to 4 months after transplant in mice³⁷. It has been further demonstrated that T and B cells derived from UCB-HSC are fully competent in immunological terms²⁵. Long-term repopulating function of HSCs in UCB was definitively confirmed by the robust contribution to multi-lineage engraftment in secondary irradiated recipients. Thus, HSCs from late fetal blood have a long-term multi-lineage repopulating ability similar to those in adult

BM, in agreement with the similar competitive repopulation capacity previously demonstrated by Harrison *et al*²².

Using a myelo-ablative conditioning regimen based on busulfan described in our previous work²⁸, we could demonstrate that also in the neonatal setting UCB has been able to repopulate the hematopoietic tissues, showing long-term multi-lineage reconstitution in mice transplanted at birth. Moreover, we showed that the number of cells derived from a single UCB sample can provide sufficient long-term repopulating ability to fully maintain a newborn recipient for at least 20 weeks.

To our knowledge, these are the first *in vivo* experiments carried out using UCBCs to perform a transplant at neonatal age. This new model of UCBT offers a potential tool to elucidate the biological features of the perinatal hematopoietic stem/progenitor cells and to develop early UCB-based therapies.

Notably, allogeneic murine late fetal or newborn blood has been transplanted in adult mouse models for prevention or treatment of autoimmune diseases such as type I diabetes and systemic lupus erythematosus^{38,39}, but never in models of genetic disorders at birth.

In Hurler disease, UCB has become in the most recent years the preferential stem cell source for affected infants and children because, in comparison with BM, this source demonstrated more immediate availability, higher donor chimerism, better enzyme recovery in blood, and superior engrafted-and-alive rates⁸. In our study, we provide evidence that neonatal UCBT in MPS-I mice allows efficient and long-term hematopoietic engraftment. Twenty weeks after neonatal UCBT, MPS-I mice with more than 50% replacement by donor-derived

hematopoiesis demonstrated near-complete normal values of biochemical parameters in visceral organs as compared with affected control mice. Indeed, the level of GAGs, which is an indicator of the disease progression, in the majority of the tissues investigated was completely normalized, confirming the efficacy of an early approach based on the infusion of UCBCs. Notably, the keratan sulfate (KS) level, which could be considered a biomarker of skeletal dysplasia in MPSs^{40,41}, was normalized after neonatal UCBT in MPS-I mice. We then focused our studies on skeletal disease, considering that it is one of the unmet clinical needs of utmost importance in transplanted MPS-I patients.

Definitely, the reconstitution of normal hematopoiesis in MPS-I mice was associated with a consistent amelioration of bone pathology, as revealed by radiographic skeletal examination. Micro-CT scans and histomorphometry remarked the impact of the high donor engraftment on the internal architecture of the femurs of transplanted mice. This could be due to enzyme delivery by hematopoietic cells close to the bone and also to tissue reconstitution by other donor-derived multipotent stem cells. Indeed, we recently demonstrated that a rare population of cells within the non-hematopoietic fraction of UCB, named cord blood-borne fibroblasts, shows *in vitro* and *in vivo* chondrogenic ability and the specific capacity of generating *in vivo* bone and a BM stroma that supports functional hematopoiesis^{42,43}. Furthermore, Uchida *et al.* demonstrated that murine UCB transplantation could fully reconstruct not only hematopoietic cells, but also mesenchymal cell lineages able to differentiate into osteoblastic cells in response to environmental specific cues^{44,45}.

Using a statistical model that separates the therapeutic effects of UCBT on MPS-I bones from the toxic effect of busulfan treatment on bones of transplanted MPS-I or WT mice²⁸, we could definitively demonstrate that neonatal UCBT reduced bone thickening in the skull, zygomatic arches, and long bone segments.

Another reported side-effect of the conditioning regimens with cytoreductive chemotherapy agent such as busulfan is bone loss due to increased bone resorption⁴⁶. In this sense, we observed a significant increase in the capacity of BM cells obtained from transplanted MPS-I mice to differentiate in TRAP-positive multinucleated osteoclasts *ex vivo*, but without achieving any actual reduction of femoral bone mineral density *in vivo*. A further assessment of bone turnover markers could be important to better elucidate the effect of conditioning on bone metabolism of MPS-I, in which the RANKL/OPG system is already altered⁴⁷.

UCB represents a promising source of stem cells for early HSCT therapeutic approaches for several diseases that can be diagnosed at birth and has several advantages such as easy and quick procurement, absence of risk to donors, immediate availability, low risk of transmitting infections, greater tolerance of HLA disparity, and lower incidence of severe GVHD^{1,2,4}. Furthermore, UCB has unique composition and biological characteristics, due to the presence of HSCs as well as a mixture of multipotent stem cells such as unrestricted somatic stem cells, mesenchymal stem cells, and endothelial colony-forming cells able to regenerate numerous tissue types with functional improvements^{3,48}. For example, the administration of human UCB cells

into MPS-III B mice decreased behavioral abnormalities and tissue pathology⁵⁰. In particular, the neuroprotective effect of human UCBCs seems to be a function of enzyme delivery and anti-inflammatory effect mediated by donor cells found throughout the brain and can be enhanced by repeated administrations⁴⁹. We do not know whether neonatal UCBT could be also effective at preventing or reverting brain pathology in MPS-I diseases. Although not investigated in our work, it is an important outstanding question that should be addressed in further studies.

Of note, UCB offers an alternative source of HSCs for gene therapy approaches, considering the possibility of collecting and storing autologous UCB at birth and reinfusing HSCs in the affected children after gene correction procedure. UCB-derived HSCs represent a particularly favorable target for gene therapy, given the reported higher gene transfer rates⁵⁰. Moreover, a neonatal gene therapy approach could help to achieve supra-normal enzyme activity in transplanted mice before disease manifestation, even in the case of low levels of chimerism. Interestingly, a pioneering study published by Simonaro *et al.* showed evidence of transduction of hematopoietic neonatal blood stem cells derived from MPS-VI cats and long-term persistence of retrovirally transduced cells into adult recipients⁵¹. Further studies would be needed to identify the best preparatory regimen suitable for transplanting affected neonates or infants⁵².

In conclusion, we demonstrated in an MPS-I mouse model the advantage of combining two factors that may allow for a better outcome in MPS-I patients: (1) early timing of the transplant and (2) the use of

UCB, which is considered at the moment the best HSC source for this disease. This study serves as a proof of concept to develop early UCB transplantation strategies for newborns affected by genetic disorders, as well as an investigational platform for novel cell and gene therapy approaches for the treatment of genetic disorders diagnosed in the neonatal period.

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AUTHOR CONTRIBUTIONS

I.A., A.P. and A.C. performed research, analyzed the data and wrote the manuscript; F.D.P., L.S., L.C., K.S. and F.K. performed research and analyzed the data; L.A. performed statistical analysis; B.G., M.E.B., M.G.V., M.R. and S.T. interpreted the data and edited the manuscript;

A.A. and A.B. edited the manuscript; M.S. designed research, interpreted the data, and wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary Methods

Antibodies

The following antibodies (eBioscience) were employed for flow cytometry analyses: anti-mouse CD45.1 PE (clone A20), anti-mouse CD45.1 APC (clone A20), anti-mouse CD45.2 PE (clone 104), anti-mouse CD45.2 APC (clone 104), anti-mouse CD45.2 PerCP-Cy5.5 (clone 104), anti-mouse CD3e PE (clone 145-2C11), anti-mouse CD45R (B220) PE (clone RA3-6B2), anti-mouse CD11b (Mac-1) PE (clone M1/70), anti-mouse Ly-6G (Gr-1) FITC (clone RB6-8C5), anti-mouse TER-119 PE (clone TER-119), anti-mouse Ly-6A/E (Sca-1) APC (clone D7), anti-mouse CD117 (c-Kit) PE (clone 2B8), and anti-mouse Hematopoietic Lineage eFluor 450 cocktail.

IDUA activity assay

Organs (spleen, liver, heart, lungs, and kidneys) were harvested at sacrifice (20 weeks), frozen on dry ice and stored at -80°C. Portions of each organ were thawed and homogenized in 500 μ L of 0.9% NaCl containing 0.2% Triton X-100 (Sigma-Aldrich) and a protease inhibitor cocktail (Sigma-Aldrich). The amount of protein in clarified supernatants of tissue homogenates was determined by Pierce BCA assay (Thermo Scientific). IDUA activity was then measured using the

fluorogenic substrate 4-methylumbelliferyl-alpha-L-iduronide (Glycosynth). 5 µg of protein were added to a solution of 0.1 M sodium formate buffer, pH 3.2, containing 8 mM D-Saccharic acid 1,4-lactone and 0.4 mM 4-methylumbelliferyl-alpha-L-iduronide. Samples were incubated at 37°C for 1 hour, then the reaction was stopped by the addition of 1 mL of 0.5 M carbonate buffer, pH 10.7. The fluorescence of the reaction product in the mix was read at 365 nm excitation and 488 nm emission wavelengths using a Tecan GENios microplate reader fluorometer (Tecan).

Glycosaminoglycans quantification in tissues

Portions of each organ collected at sacrifice were incubated overnight at 65°C with papain (Sigma-Aldrich), and then clarified for 10 min at 9391 g. GAG levels were measured using the Blyscan Sulfated Glycosaminoglycan colorimetric assay (Biocolor) according to the manufacturer's instructions. Chondroitin 4-sulfate was used as standard. Samples were read at 620 nm emission wavelength using a Tecan GENios microplate reader fluorometer (Tecan), and GAG levels were expressed as µg GAGs/mg protein in each sample.

Glycosaminoglycans quantification in plasma

At sacrifice, peripheral blood was collected in EDTA, and plasma was obtained by centrifugation at 587 g for 10 min and stored at -80°C. Ten µl of each plasma sample and 90 µl of 50 mM Tris-hydrochloric acid buffer (pH 7.0) were placed in wells of AcroPrep™ Advance 96-Well Filter Plates (OMEGA 10K, PALL Co). The filter plates were placed on the receiver and centrifuged at 2000 g for 15 min to remove free

disaccharides. The membrane plates were transferred to a fresh receiver plate. Ten μl of IS solution (5 $\mu\text{g}/\text{ml}$), 20 μL of 50 mM Tris-HCl buffer, and 10 μL of chondroitinase B, heparitinase, and keratanase II (each 2 mU/10 μL of 50 mM Tris-HCl buffer) were added onto each filter. The plate was incubated at 37°C for 5 hr and centrifuged at 2000 g for 15 min. The receiver plate containing disaccharides was stored at -20°C until injection to liquid chromatography tandem mass spectrometry (LC-MS/MS).

The chromatographic system consisted of 1260 Infinity (Agilent Technologies) and Hypercarb column (2.0 mm i.d. 50 mm, 5 μm , Thermo Electron). The mobile phase was a gradient elution from 0.025% ammonia to 90% acetonitrile in 0.025% ammonia. The 6460 Triple Quad mass spectrometer (Agilent Technologies) was operated in the negative ion detection mode with thermal gradient focusing electrospray ionization (Agilent Technologies). Specific precursor ion and product ion were used to detect and quantify each disaccharide. A m/z 354.29 precursor ion and m/z 193.1 product ion was used to detect the IS (chondrosine). Peak areas for all components were integrated automatically using QQQ Quantitative Analysis software (Agilent Technologies). The concentration of each disaccharide was calculated using QQQ Quantitative Analysis software.

Supplementary Figures and Tables

Table S1. Frequency of the different hematopoietic subpopulations in UCB and BM.

	UCB	BM	P value
T cells (CD3⁺ in CD45⁺)	2.5% (range from 1.4% to 2.8%)	3.6% (range from 2.9% to 6.0%)	0.0147
B cells (B220⁺ in CD45⁺)	4.7% (range from 3.6% to 8.7%)	10.6% (range from 7.9% to 13.2%)	0.0571
Myeloid cells (Mac-1⁺ in CD45⁺)	75.4% (range from 73.1% to 77.7%)	79.5% (range from 77.8% to 81.0%)	0.0286
Myeloid cells (Gr-1⁺ in CD45⁺)	54.3% (range from 48.6% to 56.8%)	76.0% (range from 71.6% to 78.5%)	0.0143
Erythrocytes (Ter119⁺)	67.4% (range from 61.8% to 78.9%)	2.9% (range from 0.7% to 4.1%)	0.0500

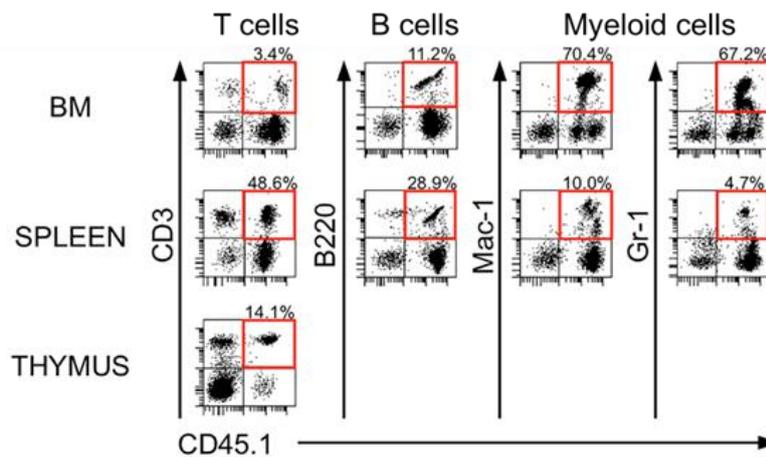
The median percentages of the different hematopoietic subpopulations in UCB and BM are reported ($n \geq 3$ for UCB, $n \geq 3$ for BM). P values calculated by 1-sided Wilcoxon test.

Table S2. Effect of conditioning on bone measurements.

	Variables		
	β_1	β_2	γ
	coeff (p value)	coeff (p value)	coeff (p value)
Skull width	4.39 (0.0003)	-2.66 (0.0151)	-3.76 (0.0012)
Zygomatic width	12.96 (<0.0001)	-1.23 (0.235)	-8.06 (<0.0001)
Femur width	6.25 (<0.0001)	-1.27 (0.2194)	-2.83 (0.0105)
Humerus width	6.96 (<0.0001)	-2.81 (0.0109)	-2.76 (0.0121)

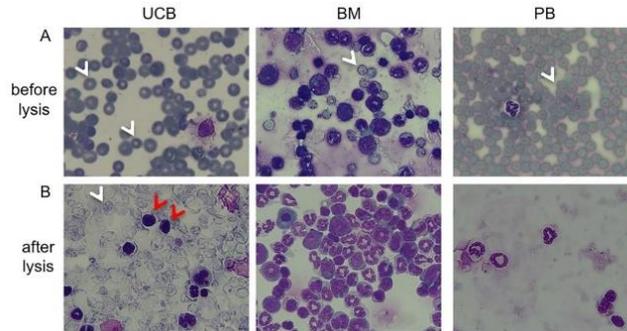
Anova regression model on categorical variables. β_1 is the effect on the parameter considered due to disease; β_2 is the nUCBT effect on WT; γ is the differential effect of nUCBT on MPS I.

Figure S1. Multi-lineage UCB-derived reconstitution within BM, spleen, and thymus of a recipient mouse at 6 months after nUCBT



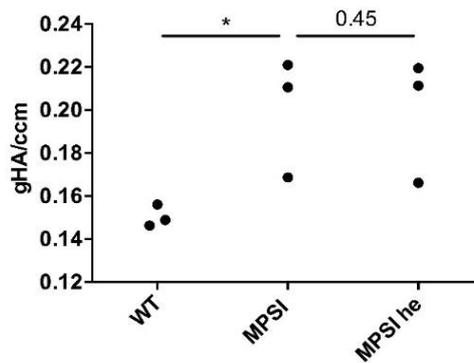
Representative lineage distribution of UCB-derived cells in the BM, spleen, and thymus of recipient mice at 6 months after nUCBT. Dot plots to determine donor-derived T cells ($CD45.1^+CD3^+$), B cells ($CD45.1^+B220^+$), and myeloid cells ($CD45.1^+Mac-1^+$ and $CD45.1^+Gr-1^+$) are shown. Percentages in total leukocytes are indicated.

Figure S2. Morphology of fetal red blood cells



Smears of day 18-fetus UCB, adult BM, and PB. Red blood cells were indicated by white arrows. Note the heterogeneity in size and color of fetal red blood cells (Magnification 50x, May Grunwald-giemsa staining). B) Cytospin preparations of the same samples after lysis. Red blood cells resistant to hypotonic lysis (white arrow) and nucleated erythroblasts (red arrows) can be found in the umbelical cord blood sample (Magnification 50x, May Grunwald-giemsa staining).

Figure S3. Bone mineral density of the femurs of WT, MPS-I, and MPS-I nUCBT-hi mice.



The graph represents the measurement of bone mineral density evaluated in the femurs of 20 weeks old WT, MPS-I, and MPS-I nUCBT-hi mice (n=3 for each group).

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CHAPTER 4

Summary, conclusions, future perspectives

Mucopolysaccharidosis type I (MPS-I) is the most frequent lysosomal disorder (LD) and is due to the inherited deficiency of alpha-L-iduronidase (IDUA) enzyme activity, which results in the accumulation of its substrates glycosaminoglycans (GAGs). This disease is systemic and clinically heterogeneous: the clinical spectrum ranges from the very severe Hurler syndrome (MPS-I-H) to the attenuated Scheie syndrome (MPS-I-S), with cases of intermediate severity known as Hurler-Scheie syndrome (MPS-I-H/S). Corneal clouding, heart disease, organomegaly, and, most of all, skeletal malformations are present in all the forms of MPS-I. Mental retardation, instead, is distinctive of Hurler syndrome, which is fatal in early childhood, thus representing the variant with the most crucial therapeutic need¹.

MPS-I has long been considered amenable to therapy by administration of exogenous IDUA enzyme, which can be uptaken by receptor-mediated endocytosis, a phenomenon known as cross-correction. The feasibility of enzyme replacement therapy (ERT) in MPS-I animal models led to successful clinical trials with the recombinant human IDUA. Currently, ERT is recommended only for patients without primary neurological disease, due to the inability of the enzyme to cross the blood-brain barrier (BBB). Furthermore, patients receiving repeated infusions of the recombinant enzyme develop antibodies which may affect the efficacy of the treatment².

Three decades ago, hematopoietic stem cell transplantation (HSCT) was introduced as a therapy for Hurler syndrome. When performed in the first years of life, HSCT is successful in alleviating many disease manifestations. Yet, the clinical consequence of HSCT for MPS-I is largely affected by graft rejection, graft versus host disease, incomplete

donor chimerism and host age at transplantation. The need for HLA-matched donors and the morbidity and mortality associated with transplant procedure limit the usefulness of this therapy, and the therapeutic impact of HSCT on the central nervous system (CNS) and skeletal manifestations is suboptimal³⁻⁵.

Yet, if transplantation is performed early in life, it does provide a more favorable clinical outcome. Treatment of MPS-I-H patients under 2 years of age with HSCT is recommended to prevent CNS involvement, and there is evidence that the sooner transplantation is performed, the better the outcome is, especially at the skeletal and neurological level^{5,6}. Recent technologies make it feasible to screen for selected types of LDs at birth, and pilot studies of newborn screening for MPS-I have been activated in several countries⁷⁻⁹. We recently offered a proof of principle of the efficacy of bone marrow transplantation in preventing skeletal abnormalities, when performed in neonatal age in the preclinical model of MPS-I¹⁰. The high levels of hematopoietic engraftment, which maintained stable in the long-term, were effective at restoring IDUA activity and clearing elevated GAGs in blood and multiple organs. Most importantly, we obtained in neonatally-transplanted mice an almost complete normalization of all bone tissue parameters, and the magnitude of improvements correlated with the extent of hematopoietic engraftment. With this work, we contributed to prove the importance of an early timing in the treatment of MPS-I.

Thinking about the application of early HSCT treatments into the clinic, another fundamental question arose regarding the best source of hematopoietic stem cells (HSCs) to transplant in MPS-I neonates. We

looked at umbilical cord blood (UCB) as a particularly fascinating source that could allow a rapid transplantation immediately after the newborn screening of the patients. Moreover, UCB-derived HSCs have unraveled to be an excellent source for transplantation in Hurler syndrome, because in the clinical practice they guaranteed higher donor chimerism and higher enzyme levels in transplanted patients, and a subsequent stabilization of bone and neurologic disease^{4,11-13}. Still, UCB-derived HSCT has never been extensively tested in a newborn setting, and the feasibility and potential of such an approach have first to be scientifically investigated in the preclinical scenery.

Beyond this point, it is clear that the amount of enzyme that transplantation can provide to an entire organism is limited by the percentage of donor chimerism and of enzyme release in blood. This is even more evident when donors are heterozygous siblings who express relatively low enzyme levels compared to healthy individuals. This is surely another issue that influences the benefits of HSCT to the CNS and skeleton^{14,15}. An answer to this matter could come from recently emerging gene therapy strategies, by which organ or cells can be modified to stably secrete high doses of functional enzyme into biological fluids. Preclinical and clinical evidences have demonstrated that autologous HSC-based gene therapy (HSC-GT) can: 1) exploit the unique properties of HSCT, capable of repopulating affected tissues (including the CNS) with myeloid cells delivering the functional enzyme; 2) further improve the therapeutic potential of HSCT by enzyme overexpression in myeloid cells; 3) reduce allogeneic HSCT side effects and morbidity/mortality. The requirement for lysosomal enzyme overexpression can be met upon lentiviral (LV)-mediated HSC

transduction and robust, long-term gene expression in their progeny, with full maintenance of stem cell properties and multi-clonal host repopulation capability¹⁶. The therapeutic efficacy of LV HSC-GT in controlling disease manifestations in visceral organs has been proved in preclinical experiments on the MPS-I mouse model, but unexpectedly high levels of lysosomal enzyme, which could not be met by the transplantation of normal donor HSCs, are required for the correction of the metabolic defect in the CNS and skeleton^{14,17}. Formal proof of the therapeutic potential of this strategy in correcting neurological disease has come from the clinical trial on metachromatic leukodystrophy (MLD), another LD with CNS involvement, and a clinical trial for the therapy of MPS-I-H children with HSC-GT is expected to start in January 2018^{14,15,18,19}. It would be chief to test in the preclinical model of MPS-I the feasibility and efficacy of autologous UCB-derived HSC-GT approach performed in neonatal age, to promote a future application of this novel therapeutic option, which could hopefully become the gold standard for a successful treatment of these patients.

The present PhD project has approached and developed many of the matters that have been discussed in the Introduction to this thesis and summarized above. The general focus of my work was the preclinical study of HSCT-based therapeutic approaches for MPS-I, that could combine an early (neonatal) time of intervention and the use of umbilical cord blood (UCB) as a source of HSCs.

We began our study collecting from the literature pre-existing knowledge on neonatal cellular and gene therapies for

Mucopolysaccharidoses (MPSs). Starting from the clinical experience, which suggests a rationale for considering the anticipation of the age of treatment either in MPSs and other inherited metabolic disorders (IMDs), we described in a review the state of the art on this matter²⁰. Indeed, an explanation of the limited efficacy of current cellular and gene therapies seems to be that the time at which they are performed is actually too late to prevent pre-existing and progressive organ damage. Thus, the clinical outcome could be more favorable if these therapies aimed at preventing, instead of correcting, disease manifestations. In the last years, several neonatal cell and gene therapy approaches have been tested in the animal models of different LDs, and they demonstrated that an early age of treatment is critical to avoid long-term clinical defects. The findings on these very early therapies, with their interesting initial results, are promising: they represent significant proves of principle for the possible application of novel treatments to MPS newborn babies. Future studies, however, are required to better assess the risks and benefits of such an early performance of these approaches. The development of cellular and gene therapy strategies to be performed in the perinatal period is now further supported by the advent of newborn screening procedures, that will allow the treatment of IMD patients before the onset of clinical signs.

The second part of this project was focused on a novel and promising source of HSCs for transplantation, umbilical cord blood (UCB). This source could be ideal for submitting babies suffering from IMDs to HSCT approaches in the very first months of life. Strategies to improve reconstitution and to enable an early therapy of these diseases by neonatal UCB-based transplantation wait to be developed in convenient

animal models. Thus, in our recently published work, we characterized murine UCB from a phenotypic and functional point of view, in comparison with adult murine bone marrow, and we verified the hematopoietic engraftment capability of this new source²¹. UCB effectively rescued adult and neonate recipients, showing a great long-term and multi-lineage repopulation capability, even in secondary transplantation setting. We then tested the therapeutic efficacy of UCB transplantation performed at birth in the MPS-I mouse model. Neonatal transplantation effectively ameliorated the biochemical and skeletal abnormalities of our disease model. We believe this study offers a proof of principle of the feasibility and usefulness of UCB-derived transplantational approaches to perform in neonatal age in MPS-I patients, which could offer them a better clinical outcome.

The continuation of this project will be performed in collaboration with Prof. Alessandro Aiuti, and will be held in the laboratories of the San Raffaele-Telethon Institute for Gene Therapy (SR-Tiget) in Milan. It will consist in the pre-clinical development of a neonatal HSC-GT approach to MPS-I. In the last months of my PhD, I moved to SR-Tiget to set the conditions for the lentiviral correction of murine MPS-I UCB cells and for their neonatal transplantation in the MPS-I model. Since this work is ongoing and obtained data are preliminary, we chose not to present them in this thesis. In this upcoming study, a procedure for the isolation of murine UCB-derived hematopoietic stem and progenitor cells (HSPCs) will be established, and these cells will be cultured *ex vivo* for LV infection. The ability of lentivirally infected UCB-HSPCs to repopulate both adult and newborn mice will be tested, and then IDUA-corrected murine MPS-I UCB cells will be transplanted in

newborn age in the MPS-I murine model. The objective of this study is to verify whether HSC-GT with IDUA-overexpressing cells could guarantee MPS-I mice a better skeletal and neurological correction. The results will tell if the autologous transplantation of gene-corrected UCB cells performed in neonatal age could solve the still unmet clinical needs of MPS-I-H patients and become their future treatment of choice.

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- **Azario I**, Pievani A, Farinelli G, Riminucci M, Gentner B, Bernardo ME, Aiuti A, Biondi A, Serafini M. *Neonatal transplantation of umbilical cord blood as a new therapeutic option for Mucopolysaccharidosis type I in the murine model*. Poster presentation. Tri-retreat meeting Telethon, May 26th-28th 2016, Rome, Italy.
- **Azario I**, Pievani A, Del Priore F, Farinelli G, Gentner B, Bernardo ME, Aiuti A, Biondi A, Serafini M. *Neonatal transplantation of umbilical cord blood as a new therapeutic option for Mucopolysaccharidosis type I in the murine model*. Oral presentation. Pathobiology of the Lysosome and Lysosomal Disease Conference, July 7th-10th 2016, Cambridge, UK.
- **Azario I**, Pievani A, Del Priore F, Santi L, Farinelli G, Gentner B, Bernardo ME, Riminucci M, Aiuti A, Biondi A, Serafini M. *Neonatal transplantation of murine umbilical cord blood and its application to the MPS-I model*. Poster presentation. European Society of Gene and Cell Therapy (ESGCT) and International Society for Stem Cell Research (ISSCR) collaborative congress, October 18th-21st 2016, Firenze, Italy.
- **Azario I**, Pievani A, Del Priore F, Antolini L, Santi L, Corsi A, Cardinale L, Gentner B, Bernardo ME, Riminucci M, Tomatsu S, Aiuti A, Biondi A, Serafini M. *Murine umbilical cord blood leads to long-term multi-lineage engraftment and reverses clinical features following neonatal transplantation in the MPS-I model*. Poster presentation. XIX Telethon scientific convention, March 13th-15th 2017, Riva del Garda (TN), Italy.

- **Azario I**, Pievani A, Del Priore F, Antolini L, Santi L, Corsi A, Cardinale L, Sawamoto K, Kubaski F, Gentner B, Bernardo ME, Valsecchi MG, Riminucci M, Tomatsu S, Aiuti A, Biondi A, Serafini M. *Neonatal umbilical cord blood transplantation halts disease progression in the murine model of MPS-I*. Oral presentation. XXI European Study Group on Lysosomal Diseases (ESGLD) workshop, September 14th-17th 2017, Lyon, France.
- **Azario I**, Pievani A, Del Priore F, Antolini L, Santi L, Corsi A, Cardinale L, Sawamoto K, Kubaski F, Gentner B, Bernardo ME, Valsecchi MG, Riminucci M, Tomatsu S, Aiuti A, Biondi A, Serafini M. *Neonatal umbilical cord blood transplantation halts disease progression in the murine model of MPS-I*. Poster presentation. Congresso dell'Associazione di Biologia Cellulare e del Differenziamento (ABCD), September 20th-23rd 2017, Bologna, Italy.
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