EUCLYD Report Summary

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Final Report Summary - EUCLYD (A European consortium for lysosomal disorders)

Executive summary:

The EUCLYD consortium has established a unique research programme to study Lysosomal storage diseases (LSDs) by developing a scientific work plan that has promoted collaborative scientific interactions between outstanding communities of basic and clinical investigators. EUCLYD has studied the mechanisms underlying the symptoms of lysosomal storage disorders to then progress towards the testing of novel therapeutic approaches with the goal of setting the scenario to directly reach patient's 'bedside'.

LSDs are caused by genetic defects that affect the synthesis or processing of lysosomal hydrolases. Therefore, a lysosomal disorder can be due to a defect in a specific hydrolase, by deficiencies in activator proteins, in the receptors or in the trafficking of enzymes. This leads to an intracellular accumulation of a variety of undegraded cellular substrates, including sphingolipids, glycosaminoglycan and glycogen. Forty to fifty LSDs are presently known. As a group they occur with an estimated frequency of 1 / 2 000, but each of them is rare.

The four specific LSDs chosen in this research programme (Pompe disease, Gaucher disease, MPS VI, MSD) have each unique characteristics, they all differ for the type of stored material and for the involvement of different tissues. This results in different challenges in the treatment of patients and has therefore implications for the choice of specific therapeutic strategies.

The overall goal of the project has been to:

i) study specific lysosomal diseases;  
ii) understand the pathophysiology;  
iii) determine the natural history of these rare diseases in order to develop tools to measure therapy efficacy; and  
iv) test and develop novel therapeutic approaches utilising appropriate animal models of human disorders.

The final objective of the EUCLYD consortium has been to shed light on the course and pathophysiology of LSDs and to develop proof-of-principle tests on novel therapeutic approaches.

According to the experimental plan, the consortium has studied the pathophysiology of Multiple sulfatase deficiency (MSD), Gaucher disease, Pompe disease and mucopolysaccharidosis (MPS VI) (WP1). These studies have unveiled the basic mechanisms to explain disease phenotype. This knowledge has also allowed developing therapies to contrast the devastating effects of accumulation, and to understand whether and when therapies should be devised. In addition, research has been focused on the natural history of Gaucher disease, Pompe disease and MPS VI (WP2). The study of the natural history of these disorders is essential to evaluate the effectiveness of therapy. Novel therapeutic strategies for LSDs consist of the application of small molecules that act as chaperones to increase the residual activity of the lysosomal enzymes, known as enzyme enhancement therapy (WP3) or small molecules that inhibit substrate synthesis, known as substrate reduction (WP4). Finally,
the consortium has tested and developed new protocols utilising Adeno-associated Virus (AAV) vectors to directly administer the wild-type gene into a factory organ taking advantage of normal lysosomal enzyme trafficking and a phenomenon known as 'cross-correction' (WP5).

Individuals with lysosomal disorders, which largely affect children in the first decade of life, will greatly benefit from continual refinement and optimisation of the current therapy, as well as from the development of new treatment modalities that offer improvements in efficacy, cost, safety and availability. By developing an experimental plan to understand the basis, the natural history and to test potential therapies, the EUCLYD consortium has provided an enormous impact on the health of children who are being devastated by this type of progressive, debilitating and often lethal group of disorders.

Project context and objectives:

Lysosomes are membrane-enclosed compartments, filled with hydrolytic enzymes that are used for the degradation of macromolecules. Proteins and other substrates are delivered to the lysosomes by various pathways including endocytosis, and autophagy, a pathway utilised by the cell for the disposal of obsolete parts. Many steps are necessary for the correct synthesis and processing of lysosomal enzymes.

LSDs are caused by genetic defects that affect the synthesis or processing of lysosomal hydrolases. Therefore, a lysosomal disorder can be due to a defect in a specific hydrolase, by deficiencies in activator proteins, in the receptors or in the trafficking of enzymes. This leads to an intracellular accumulation of a variety of undegraded cellular substrates, including sphingolipids, glycosaminoglycan and glycogen. Forty to fifty LSDs are presently known. As a group they occur with an estimated frequency of 1 / 2 000, but each of them is rare.

The EUCLYD consortium has established a unique research programme to study LSDs by promoting collaborative scientific interactions between outstanding communities of basic and clinical investigators. To improve human health, scientific discoveries must be translated into practical applications. EUCLYD has focused on the study of the mechanisms underlying the symptoms of LSDs to then progress towards clinical applications through the development of novel therapeutic approaches with the final goal of moving from 'bench to bedside'.

Amongst the LSDs presently known, EUCLYD has focused on: Gaucher disease, Pompe disease, MPS VI and MSD. The four specific LSDs chosen in this research programme have each unique characteristics, they all differ for the type of stored material and for the involvement of different tissues. This results in different challenges in the treatment of patients and has therefore implications for the choice of specific therapeutic strategies.

The overall goal of the project has been to:
1. to study specific lysosomal diseases;
2. understand the pathophysiology;
3. determine the natural history of these rare diseases in order to develop tools to measure therapy efficacy; and
4. test and develop novel therapeutic approaches utilising appropriate animal models of human disorders.

The specific objectives have been to:

1. Characterise the pathophysiology of MSD, MPS VI and PD to identify disease mechanisms and markers. This characterisation has been done utilising specific markers and assays that have allowed to quantify and verify the mechanisms studied.
2. Determine factors that modify clinical diversity between PD patients bearing the same GAA haplotype. This has been determined by using specific assays.
3. Identify tissue-pathology markers as determinants of PD stage. The success of therapeutic intervention seems largely determined by the stage of disease at start of treatment. Furthermore, these markers could help in predicting efficacy of
therapy.

4. Characterise the natural history of a subset of LSDs in order to have the correct tools to evaluate treatment efficacy and to obtain information to develop standardised European protocols for the different LSDs, starting with Pompe disease and MPS VI. This has allowed collecting homogeneous information on a large number of patients and, ultimately, has been useful to further validate the information available until now on the therapeutic outcome.

5. Explore the use of enzyme enhancement by pharmacological chaperones, for the treatment of Pompe disease.

6. Determine the effect of miglustat and other substrate inhibitory analogues, such as the morpholino compounds, on de novo biosynthesis of glucosylceramide and determine whether their action serves as an inhibitor of cytokine release that accompanies Gaucher disease.


The final objective of the EUCLYD consortium has thus been to shed light on the course and pathophysiology of LSDs and to develop proof-of-principle tests on novel therapeutic approaches. Therapeutic approaches can only be tested if disease mechanisms are understood to identify pathogenetic markers and natural history to evaluate therapeutic outcome and long-term therapy efficacy.

A formal evaluation of therapy efficacy in lysosomal diseases is difficult due to the marked variability of clinical phenotypes. Enzyme replacement therapy and enhancement therapies have been developed to treat these disorders, albeit with variable results. New treatment strategies have been considered for patients with LSDs including gene therapy, substrate reduction therapy and chaperone therapy. Individuals with lysosomal disorders will greatly benefit from continual refinement and optimisation of the current therapy, as well as from the development of new treatment modalities that offer improvements in efficacy, cost, safety and availability.

Finally, it is important to stress that LSDs are characterised by a progressive, highly debilitating or lethal course, causing severe handicap in patients resulting in significant social burden. Moreover, many of these diseases affect children in the first decade of life. By developing an experimental plan to understand the basis, the natural history and to test potential therapies, the EUCLYD consortium has provided an enormous impact on the health of children who are being devastated by this type of progressive, debilitating and often lethal group of disorders.

Project results:

The interest of the EUCLYD consortium is the study of LSDs (LSDs), a heterogeneous group of disorders that encompass around 50 distinct metabolic diseases. The incidence of LSDs as a group is 1 / 2 000, but, taken individually, each disease is very rare and many have an incidence of less than 1:100 000. However, one important peculiarity of LSDs is that data indicate that the basis of the diseases and pathogenetic mechanisms of the symptoms may have common grounds; therefore, the study of a few more relevant examples, such as Gaucher disease, Pompe disease, MPS VI and MSD, may have huge implications for the entire group of disorders.

According to the experimental plan, the consortium has studied the pathophysiology of MSD, Gaucher disease, PD and MPS VI (WP1). These studies have unveiled the basic mechanisms to explain disease phenotype. This knowledge has also allowed developing therapies to contrast the devastating effects of accumulation, and to understand whether and when therapies should be devised. In addition, research has been focused on the natural history of Gaucher disease, Pompe disease and MPS VI (WP2). The study of the natural history of these disorders is essential to evaluate the effectiveness of therapy. Novel therapeutic strategies for LSDs consist of the application of small molecules that act as chaperones to increase the residual activity of the lysosomal enzymes, known as enzyme enhancement therapy (WP3) or small molecules that inhibit substrate synthesis, known as substrate reduction (WP4). Finally, the consortium has tested and developed new protocols utilising AAV vectors to directly administer the wild-type gene into a factory organ taking advantage of normal lysosomal enzyme trafficking.
and a phenomenon known as 'cross-correction' (WP5).

The main scientific and technological results obtained by the EUCLYD consortium are outlined below and in the following pages.

LSD: pathophysiology

In eukaryotes, most of the cellular clearing processes occur in a specialised organelle, the lysosome, which receives and degrades macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways. Lysosomes contain proteins with different functions, including hydrolases, transmembrane proteins involved in membrane fusion and transport, and a complex proton pump that is required for the acidification of the organelle. Importantly, the lysosome is involved in numerous diseases including LSDs, characterised by lysosomal dysfunction and defects in intracellular trafficking. This leads to intracellular accumulation of a variety of undegraded cellular substrates, including sphingolipids, glycosaminoglycans and glycogen. In most cases these disorders are characterised by a progressive, highly debilitating or lethal course, associated with neurodegeneration, causing severe handicap in patients and resulting in a significant social burden.

As the degradative requirements of the cell vary depending upon tissue type, age and environmental conditions, it is reasonable to expect the existence of systems that allow regulation of lysosomal function. Genetic programs that control organelle biogenesis and function may mediate such coordination and facilitate the coordination of complex functions, such as cellular clearance. We discovered a gene regulatory network that controls lysosomal biogenesis and function. The transcription factor TFEB acts as a modulator of the CLEAR network and is physiologically activated by lysosomal storage. Upon activation, TFEB translocates into the nucleus, binds to CLEAR target sites in the promoter of lysosomal genes, and induces lysosomal biogenesis (Sardiello et al., 2009). We then demonstrated that TFEB controls lysosomal exocytosis (Medina et al., in press). During lysosomal exocytosis, a Ca2+-regulated process, lysosomes are docked to the cell surface and fuse with the plasma membrane (PM), emptying their content outside the cell. This process has an important role in secretion and PM repair. We showed that TFEB regulates lysosomal exocytosis. TFEB increases the pool of lysosomes in the proximity of the PM and promotes their fusion by raising intracellular Ca2+ levels through the activation of the lysosomal Ca2+ channel called MCOLN1. Induction of lysosomal exocytosis by TFEB overexpression rescued pathologic storage and restored normal cellular morphology both in vitro and in vivo in LSDs (LSDs, MSD). Our data indicate that lysosomal exocytosis may directly modulate cellular clearance and suggest a novel therapeutic strategy for disorders associated to intracellular storage.

Pompe disease: pathophysiology and natural history

With an estimated incidence of 1 in 40 000 Pompe disease is indeed a rare disease. The pathogenesis is well understood in the sense that the disease is caused by sequence variations in the gene coding for acid alpha-glucosidase. Some of these variations are harmless but others lead to loss of function. Acid alpha-glucosidase is one of the estimated 50 enzymes that together secure lysosomal functioning. Proper function of acid alpha-glucosidase is essential for the degradation of glycogen that has entered the lysosomes by autophagy, a process by which cells and tissues renew themselves. Shortage or complete lack of acid alpha-glucosidase leads to storage of glycogen and cellular damage. As muscle cells are most vulnerable to glycogen storage, acid alpha-glucosidase deficiency leads to muscle weakness and wasting, predominantly affecting the proximal muscles and the pulmonary function of affected individuals. Infants without any acid alpha-glucosidase activity succumb in the first year of life due to cardio-respiratory failure.

The European collaborative effort of EUCLYD has enabled to address the many aspects of Pompe disease in parallel: i.e. the underlying mutations and their effect, renewal of diagnostic procedures and introduction of methods to estimate the ‘CRIM status’ and the antibody titer evoked by enzyme therapy, the role of autophagy, the role of muscle fibre type involvement, the natural course including survival, muscle function, pulmonary function, hearing and cardiac function in children, the
impact of disease modifying factors, the effect of enzyme replacement therapy, the corrective effect of chaperones on pathogenic forms of acid alpha-glucosidase and the additive effect of chaperones when administered in combination with enzyme replacement therapy.

The EUCLYD consortium has successfully characterised multiple cellular abnormalities in Pompe disease cultured fibroblasts (including intralysosomal storage of glycogen, increased number of multivesicular bodies, activation of autophagy, expansion of the Golgi apparatus), and identified abnormalities in the trafficking and recycling of the cation-independent mannose-6-phosphate receptor (CI-MPR). All these abnormalities appeared to be more prominent in severe and intermediate PD fibroblasts, apparently correlating with disease severity.

During the three years of the EUCLYD programme the number of patients cared for by the clinicians of the consortium (EMC, UMC) has substantially increased, mainly as a result of the approval and market introduction of enzyme replacement therapy (Myozyme) in 2006. Managing the increasing number of patients has required the design of a study protocol and a package of clinical assessments to secure the systematic follow-up of all patients. All protocols are now in place to diagnose the patients at an early stage of disease with improved clinical and laboratory procedures. EMC, in particular, has established an assay to measure acid alpha-glucosidase activity in white blood cells, and other methods to measure the activity in dried blood spots.

The making of blood smears and staining of the lymphocytes with periodic acid schiff reagent - to demonstrate glycogen storage - was shown to assist in the diagnosis of Pompe disease patients with both very early onset (< 1 month of age) as well as very late onset (> 70 years of age) of symptoms. This finding was unexpected since patients with late onset of symptoms do not store glycogen in their cultured skin cells while patients with early onset of symptoms do. The difference between glycogen storage in lymphocytes versus skin cells might lay in the differential expression of the acid alpha-glucosidase gene in these different cell types although work is still in progress to fill this knowledge gap (the planned experiments will be completed with financial support of EMC within the end of 2011). Hopefully, the new generation sequencing technology that has meanwhile become available will also shed light on the genetic factors that substantially modify the clinical course of Pompe disease. The latter relates to the observation in our continuously growing patient population that patients with the 'same' acid alpha-glucosidase genotype can have a widely differing course of disease with regard to age of onset. Skin cells of 12 patients with very early onset and 12 patients with very late onset of symptoms have been collected and are presently being analysed for acid alpha-glucosidase activity, glycogen storage and mRNA expression level of all expressed genes. These latter activities enabled by EUCLYD hopefully will guide to new ways of therapeutic intervention.

The systematic follow-up of large numbers of patients with Pompe disease over the past three years has enabled us to accurately document the natural course of Pompe disease and the effects of enzyme replacement therapy.

By mutation analyses of the GAA gene we characterized the genotype in all enrolled patients (UMC, N=48) and identified some genotype-phenotype correlations (Herzog et al., in preparation). Furthermore our studies contributed to the description of dilated arteriopathies in Pompe disease, an unprecedented disease complication. Additionally, we recognised, by interim analysis, high prevalence of respiratory insufficiency and abnormal breathing during sleep due to diaphragmal weakness years prior to diagnosis in new diagnosed adult Pompe patients. To increase awareness and to study the natural course of diaphragmal weakness in adult Pompe patients a cooperative study in the 3 largest German Pompe centres has begun. Timely diagnosis will improve the long-term outcome by optimising respiratory management and by early initiation of enzyme replacement therapy.

Study results in the infantile Pompe cohort have raised the issue of antibody formation against rhGAA. As a consequence, we studied the impact of antibody formation on safety and efficacy of rhGAA in infantile Pompe patients. We also developed new methods to monitor antibody formation against the therapeutic enzyme and to establish the "CRIM status" of severely affected infants. ‘CRIM’ stands for any small amount of acid alpha-glucosidase that is detectable in the patient's tissues. CRIM-negative patients are more prone to develop high antibody titeres during enzyme therapy than CRIM-positive patients. We
investigated the role of CRIM status in 11 of our infantile patients and confirmed previous findings that a CRIM-negative status is a poor prognostic factor for successful clinical outcome, but the correlation between the height of the antibody titer (the height of the immune response) and the clinical outcome is not very strict. An incidental finding of a very high antibody response in an adult patient receiving enzyme replacement therapy has led to in-depth analysis of the effect of antibody formation as a result of enzyme replacement therapy. It has resulted in an understanding of what antibody formation and low versus high antibody titers actually means for the successful treatment of patients.

The newly developed methods for determining the CRIM status and the height of antibody titers are now routinely applied as diagnostic tool and for the follow-up of patients during treatment. EMC offers diagnostic services for Pompe disease at the level of enzyme assay and DNA analysis to many European and non-European countries; CRIM tests, antibody testing and analysis of the functional effect of newly discovered acid alpha-glucosidase mutations are included.

As to the natural course of Pompe disease, in the context of EUCLYD and related programmes, a study was performed to gather information that is yet lacking on the survival of adults with Pompe disease. This study, which was carried out in a large sample of individuals (268 adults), was completed and showed for the first time that untreated patients have a higher mortality than that observed in the general population. The levels of disability and handicap / participation are the most important factors associated with mortality.

The condition of 94 adult patients who did not receive enzyme replacement therapy was monitored during their regular visits to hospital (EMC). At study entry, the mean age of these patients was 50 years old (range 25-75 yrs). The skeletal muscles of the proximal lower extremities and the trunk were most affected, and the muscle weakness increased by 1.3 % to 2.6 points / year. The forced vital capacity in sitting position deteriorated by 1% point per year (p=0.06) and in supine position by 1.3 % points per year (p=0.02). A substantial number of patients had less well-known features of Pompe disease such as ptosis (23 %), bulbar weakness (28 %), or scapular winging (33 %). Hearing loss was studied in 58 affected adults using tympanometry and pure-tone audiometry and appeared to be no feature of Pompe disease in adults. Fatigue was a frequent finding. The bone mineral density (BMD) was measured in 36 adults and 10 children with Pompe disease and was found to be significantly lower than in the reference healthy population.

Pompe disease: enzyme replacement and enzyme enhancement therapy. The characterisation of the CI-MPR pathway in PD cells has important clinical implications. For PD, like for some of the most prevalent LSDs, enzyme replacement therapy with recombinant human alpha-glucosidase (rhGAA) is the only approved treatment. Enzyme replacement is based on the concept that recombinant lysosomal hydrolases can be internalised by cells and tissues through the mannose or mannose-6-phosphate receptor (MPR) pathways and are ultimately delivered to lysosomes, where they replace the function of the defective hydrolases. Thus, the integrity of the mannose-6-phosphate pathway is a requisite for the efficacy of enzyme replacement. In Pompe disease, enzyme replacement efficacy is variable in different patients and it is known that some tissues (like the skeletal muscle) are refractory to this treatment. The finding of a deranged CI-MPR function in PD cells may provide an explanation for the variable response to enzyme replacement (and indicate possible therapeutic strategies directed toward the correction of these abnormalities).

Sixty-nine patients were monitored before and after the start of enzyme replacement therapy (EMC). In this group of patients with a treatment duration of 2-4 yrs the muscle strength improved by 1.4 to 4.0 points per year. The pulmonary function (FVC) in sitting position (N=62) stabilised, but the FVC in supine position (p < 0.05) (N=54) declined by 1 % per year. The best responding patients were those of younger age, with shorter disease duration and with the least disease severity. It is certain that the occurrence of high antibody titers hampers enzyme therapy, but it is not fully understood why some patient generate high antibody titers while others don't.

In addition, we have provided the proof-of-principle for the use of pharmacological chaperones as a potential therapy of PD. Enzyme enhancement therapy is based on the concept that loss-of-function diseases are often due to missense mutations
causing misfolding and degradation of catalytically competent enzyme proteins. A partial rescue of enzyme activity may be obtained by active site-directed competitive inhibitors, that can improve folding and stability of mutated proteins with altered conformations by acting as folding templates.

We demonstrated that two imino sugars, 1-deoxynojirimycin (DNJ) and its alkylated derivative N-butyl deoxynojirimycin (NB-DNJ, Miglustat) are effective in enhancing GAA residual activity in fibroblasts from PD patient carrying specific mutations of the GAA gene and act as pharmacological chaperones. This approach, however, has important limitations as it may be applicable only to patients with specific GAA gene mutations. We estimated that about 10-20 % of Pompe disease patients may be amenable to enzyme enhancement therapy.

This limitation could be apparently overcome according to the results obtained in another study, in which we showed that chaperones are not only effective in enhancing mutated GAA activity, but can also potentiate the efficacy of enzyme replacement. Co-administration of rhGAA and NB-DNJ to Pompe disease fibroblasts resulted in improved trafficking of the recombinant enzyme to lysosomes, improved maturation and increased intracellular GAA activity. Thus, these results indicate a synergistic effect between pharmacological chaperones and enzyme replacement. The synergistic effect between enzyme enhancement therapy and enzyme replacement was confirmed in a certain number (> 10) of PD cell lines. In addition, preliminary and unpublished results obtained by the partners of the EUCLYD consortium have demonstrated that this synergy between enzyme replacement and enzyme enhancement therapy is also observed in another lysosomal storage disease, Fabry disease.

The implications of the results obtained in our studies are:
- the use of pharmacological chaperones should not be restricted only to patients with responsive mutations, but may be extended to any Pompe disease patient on enzyme replacement, with obvious advantages for the cure of patients;
- our data provide the rationale for the use of combination therapeutic protocols in the treatment of Pompe disease (and possibly other LSDs). Pompe disease, like most lysosomal diseases, is a complex disorder, characterised by generalised glycogen storage and multi-organ involvement. It is reasonable to think that approaches based on the combination of different therapies may be more effective in correcting all the aspects of the disease.

These hypotheses are now being translated into an Italian multicentre clinical trial, beyond the scope of the EUCLYD programme.

Gaucher disease: pathophysiology and natural history

Gaucher disease is an autosomal recessive LSD due to the deficiency of beta-glucocerebrosidase. Gaucher disease is characterised by the storage of sphingolipids (glucosylceramide, GlcCer) primarily within cells of phagocyte origin (‘Gaucher cells’). Gaucher disease is the less rare LSD, with an estimated incidence ranging between 1:57 000 and 1.16:100 000 (although it is significantly higher in specific populations as Ashkenazi Jewish descent). The clinical phenotype of the disease includes visceral (hepatosplenomegaly), hematologic (cytopenia), and skeletal (osteolytic lesions, bone crises, femoral Erlenmeyer flask deformity) manifestations.

Gaucher patients under the care of clinics supervised by the EUCLYD partners are now registered in independent databases, including patients receiving substrate inhibitors. The consortium has assembled a clinical database of Gaucher patients, which can be interrogated for further evaluation of licensed substrate inhibitor treatment with miglustat. Plasma samples are available for analysis from several groups, including those receiving no treatment, enzyme replacement therapy and substrate reduction therapy with miglustat. Their clinical behaviour and effects of miglustat have been recorded and several toxic unwanted effects have been experienced, which are currently being annotated for documentation and reporting in the literature (see also below).
Concerning patient-reported outcome measures, the EuroQol 5D (EQ5D) quality of life summary measure is significantly lower (median 0.679) in those patients with a history of osteonecrosis, compared with those with no such history, (median 0.796 p<0.01). The health-state score was also found to be significantly lower (median 0.626) in those who had suffered a fragility fracture, representing osteoporosis, than in those who had not (median 0.796 p<0.001).

The consortium has completed studies to investigate the relationship between circulating chemokines and cytokines and the most disabling complication affecting life quality of patients with Gaucher disease - osteonecrosis. The concentration of the chemokine biomarker, PARC / CCL18, appears to be significantly greater in patients with a history of osteonecrosis (p<0.01) and there is a significant correlation between the number of anatomical sites and the elevation of this chemokine. Subsequent studies have been completed to quantify cytokines / chemokines and many of these molecules have been found to be elevated in Gaucher disease before treatment.

We have completed multiplex assays in samples obtained from a cohort of 100 adults with Gaucher disease attending 3 referral centres who have been independently evaluated for quality of life and disease-related complications as part of a structured assessment compounding an independent clinical bone registry.

Gaucher disease: enzyme replacement, gene therapy, substrate reduction The consortium has completed a study of the osseous manifestations of adult Gaucher disease in the mature era of enzyme therapy. While this treatment has the capacity to prevent many of the emergent manifestations of Gaucher disease, and may reverse the haematological and visceral effects, its benefit on the skeleton has yet to be determined and has been the subject of the work supported by EUCLYD. In 9 adults with Gaucher disease who had received enzyme replacement therapy from childhood out of 100 surveyed, one patient presented with established bone disease (osteonecrosis) and was treated. A second patient with an episode of osteonecrosis aged 5, was treated from 9 years. Of the remaining 7, none had osteonecrosis either before or after treatment, and of the total number of 9, none experienced osteonecrosis after the start of treatment. These findings strongly suggest that early introduction of therapy is critical and all these patients were free of active disease in the skeleton and had excellent quality of life as mature adults.

Concerning the development of gene therapy for Gaucher disease, hematopoietic stem cell-based gene therapy offers the possibility of permanent correction for genetic disorders of the hematopoietic system. However, optimisation of present protocols is required before gene therapy can be safely applied as general treatment of genetic diseases. The EUCLYD consortium used a mouse model of type 1 Gaucher disease to demonstrate the feasibility of a low-risk conditioning regimen instead of standard radiation, which is associated with severe adverse effects. We first wanted to establish what level of engraftment and glucosylceramidase (GCase) activity is required to correct the pathology of the type 1 GD mouse. Our results demonstrate that a median wild-type (WT) cell engraftment of 7%, corresponding to GCase activity levels above 10 nmoles / hour and mg protein, was sufficient to reverse pathology in the bone marrow and spleen in the GD mouse. Moreover, we applied nonmyeloablative doses of busulfan as a pretransplant conditioning regimen and showed that even WT cell engraftment in the range of 1%-10% can confer a beneficial therapeutical outcome in this disease model. Taken together, our data provide encouraging evidence for the possibility of developing safe and efficient conditioning protocols for diseases that require only a low level of normal or gene-corrected cells for a permanent and beneficial therapeutic outcome.

To generate safer gene therapeutic approaches, we have generated several self-inactivating lentiviral vectors that are less likely to cause serious insertional mutagenesis effects by upregulating cancer-promoting genes. These vectors contain the glucocerebrosidase gene driven by the SFFV promoter (strong promoter control vector) and vectors containing either the CD11b promoter (tissue-specific for myeloid cells) or the PGK promoter (a relatively weak promoter in hematopoietic cells. All vectors contain the GFP gene, which is expressed through an IRES between the two genes. These vectors have been constructed and can transduce primary murine hematopoietic cells from type 1 Gaucher mice. All vectors increase the expression of the glucocerebrosidase enzyme to levels high enough to correct the enzyme deficiency when the transduced cells are grown in vitro. Due to difficulties in inducing the phenotype, the in vivo gene therapy studies have been delayed by
one year. They are all in progress now and we expect to have findings one year from now that show enzyme correction in Gaucher mice in vivo. Similarly, studies to analyse insertional mutagenesis will be finished at the same time.

Several substrate inhibitors have been investigated by members of the consortium for Gaucher disease, including rhodamine B, to restrict the biosynthesis of the macromolecular substrates stored in lysosomes. Imino sugars (miglustat) are in current licensed use in Gaucher disease; an unrelated inhibitor, GENZ112638, eliglustat tartrate, is in clinical trial for Gaucher disease and is a ceramide analogue. Unfortunately, the potency of rhodamine and other related compounds under exploration as inhibitors of the biosynthesis of glycosaminoglycans was insufficient for further investigation clinically with inhibitory actions in the high micromolar or low minimolar range.

Full clinical laboratory tests have been conducted in patients receiving miglustat in both centres (UCAM, UMC). The effects of the treatment on disease behaviour as reflected by chemokines haematopoietic indices and visceral size have been studied. In several instances miglustat proved unsatisfactory and a full report on the role of this agent in both stable and unstable patients is shortly to be submitted.

The trial of GENZ-112638 in the authentic model of Gaucher disease in genetically modified mice induced with the interferon analogue, poly[I:C] has been completed. Preliminary analysis has shown marked reduction of plasmaglucoceramide at all 3 doses of the substrate inhibitor in the test, compared with the matched controlled diet. Further studies of the effect of the inhibitor are awaiting mass spectroscopic analysis but all relevant tissues from a large number of experimental animals have been collected.

Further collaborations among members of the EUCLYD consortium are under way to improve the expression of the Gaucher phenotype for further study and a supply of second-generation inhibitors has been arranged with the Genzyme Corporation for further detailed characterisation and study. Unfortunately, the full-blown Gaucher phenotype was not evident in the inducible model and a programme of further collaborative breeding with scientists in ULUND is in progress to ensure more profound glucocylceramidase sufficiency in the study animals.

However, a unique phenotype in the long-term survivors harbouring the Cre gene recombinase gene (potential Gaucher) has been compared with wild-type, mutant heterozygotes and Cre negative animals, irrespective of the inducing agent. Several animals show massive splenomegaly and lymphoid aggregates indicative of spontaneous tumours. These animals are actively under investigation as a model of B-cell malignancy, which complicates adult Gaucher disease in humans. Within a few weeks preliminary data on whether substrate inhibitors suppress this phenotype will be available.

MPS VI: pathophysiology and natural history

MPS VI is caused by deficient arylsulfatase B (ARSB) activity resulting in lysosomal storage of glycosaminoglycans (GAGs). MPS VI is characterised by dysostosis multiplex, organomegaly, corneal clouding, and heart valve thickening. The incidence of MPS VI is even lower than that of Pompe disease.

Our studies on the pathogenesis of MPS VI have indicated that non-lysosomal degradation pathways are impaired both in MPS VI cell lines and in MPS VI rat tissues as a result of dermatan sulfate accumulation. These pathways can be both targets of new experimental therapies and biomarkers for follow-up of existing treatments.

The clinicians of the EUCLYD consortium have developed protocols for the diagnosis and clinical follow-up of patients with this disease as has been done for patients affected by Pompe disease. The systematic collection of data by EMC has led thus far to the description of the cardiac problems, the collection of genotypes, the production of antibodies for CRIM testing (as in Pompe disease), the development of an ELISA assay to measure the height of the immune response against enzyme replacement therapy, the assessment of antibody titers in 9 patients receiving enzyme therapy, and the documentation of the
clinical effects of therapy over a period of 1.5 years. With the help of a newly designed questionnaire (MPS Survey) we have also assessed the quality of life of patients with MPS VI, like we did for Pompe disease, in a collaborative effort with the Dutch patient association (VKS). All these activities performed at EMC and by its partners in the context of EUCLYD have led to an upgrading of the quality of care in and outside Europe for patients with these rare LSDs.

MPSVI: enzyme replacement (ERT), gene therapy, substrate reduction

The first years with enzyme replacement therapy in MPS VI have demonstrated that patients benefit from ERT regarding quality of life, lung function and endurance. However, we described disease complications in relation to the phenotype, which did not respond to enzyme replacement therapy. Early diagnosis and timely intervention of these complications, such as craniocervical stenosis or heart valve abnormalities, are essential for optimal management. Our studies have focused on a diagnostic algorithm to detect craniocervical stenosis timely before irreversible changes occur.

In addition, we found that onset of craniocervical stenosis as well as heart valve findings vary substantially between patients with classical and attenuated / slowly progressive phenotype. Additional to mutations in the ARSB-gene we identified severity of growth retardation and GAG excretion as clinical predictive factors for the phenotype.

In addition, we have set-up a gene therapy protocol in animal models of MPS VI based on the systemic delivery of AAV vectors and on the conversion of the liver in a factory for long-term sustained release of arylsulfatase B to deficient tissues. We have shown that this strategy results in significant improvement of the biochemical, pathological, skeletal and motor function anomalies in MPS VI rats and cats. In MPS VI rats, as results of the null ARSB mutation causing the disease, upon AAV administration animals develop an immune response to the recombinant ARSB which limits therapeutic efficacy and can be prevented by co-administration of immune-suppressive drugs. This does not occur in MPS VI cats bearing missense ARSB mutations and expressing an inactive ARSB enzyme. Indeed, in MPS VI cats a single intravascular administration of AAV8 results in ARSB expression up to 3 years after vector delivery, the last time point of the analysis. This bodes well towards the clinical development of gene therapy for MPS VI.

Finally, we have provided preliminary evidence supporting a novel therapeutic approach based on substrate reduction for the treatment of mucopolysaccharidoses (MPS). This approach is also based on the use of small molecule drugs (like pharmacological chaperones) and has the potential to overcome some of the limitations of ERT, such as the insufficient distribution of recombinant enzymes in tissues and organs and the need for frequent intravenous infusions.

As substrate-reducing agent, we tested a member of beta-D-xylosides, a class of variably substituted compounds that in earlier studies have been shown to prime GAG polymerisation independently of core proteins, by serving as acceptors in the first galactosylation and the subsequent elongation steps. We showed that this drug can reduce glycosaminoglycan (GAG) and proteoglycan (PG) synthesis in cultured fibroblasts from patients with different MPS (MPS IIIA, II and VI).

We also tested the effects of beta-D-xylosides on the synthesis of PGs in the mouse model of MPSIIIA that recapitulates the phenotype of the disease. In a preliminary short-term test 1 month-old mice were treated with 50 mg / day for 2 months intraperitonealy. During treatment with the beta-D-xyloside the mice did not show clinically overt signs of toxicity. At the age of 3 months the animals were examined, sacrificed, and GAG content of different tissues was assayed and compared to those obtained in 3 month-old MPSIIIA untreated mice. In xyloside-treated mice GAG content was decreased in the liver and brain.

MSD: gene therapy

The landmark of MSD deficiency is the severe or lack of activity of the entire repertoire of endogenous sulfatases. This is due to genetic defects affecting the sulfatase modifying factor 1 (SUMF1) gene. SUMF1 is a so-called modifier gene whose key activity is to activate sulfatases through an enzymatic activity. As a consequence, important metabolic functions of cells are
impaired, eventually leading to the abnormal storage of toxic compounds (glycosaminoglycans; GAGs) within the sub cellular organelles called lysosomes. Gene therapy holds promise to correct this pathological lysosomal storage based on the correction of the underlying DNA defects affecting SUMF1. One promising system relies on gene transfer with modified viral vectors of the correct copy of the gene in affected cells. In addition, since SUMF1 is expressed in any tissue of the body one major challenge to cure the disease is to transfer the correct copy of SUMF1 efficiently in the whole body. Previous studies have indicated the so-called viral vector based on AAV type 9 as a very efficient delivery vehicle. Based on this premise we generated AAV9 vectors containing the SUMF1 gene and administered them into neonatal Sumf1-/- mice, early during disease progression. As a result, we showed that visceral organs exposed to the vector were efficiently infected by AAV9-CMV-SUMF1 vector, and this, in turn, resulted in the global visceral activation of sulfatases. The activation of sulfatases led to clearance of GAGs accumulation within distinct organs.

In addition, we showed that administration of AAV9-CMV-SUMF1 in neonatal MSD mice resulted in significant amelioration of the inflammatory status of visceral organs including the liver and rescue of behavioural motor defects affecting bone-joints.

In summary, this proof-of-concept study provides converging evidence at different levels of analysis that rAAV9 vector mediated SUMF1 injection can significantly ameliorate the visceral phenotype of a mouse model of MSD, which is perhaps the most severe lysosomal storage disorder affecting the whole body.

Potential impact:

The EUCLYD consortium has aimed at establishing a unique research programme to tackle LSDs by putting together a consortium that has promoted collaborative scientific interactions between the outstanding communities of basic and clinical investigators. The consortium and scientific programme was thought to allow the interaction between basic scientists and clinicians: basic science observations to provide clinicians developing therapeutic approaches with new tools for use in patients and for assessment of their impact, and clinical researchers to make novel observations regarding natural history, mutant-phenotype correlations on the nature and progression of disease that often stimulate basic investigations.

The potential impact of the work obtained by EUCLYD on the understanding and potential treatment of rare diseases due to metabolic disorders such as lysosomal storage dysfunction is described below:

- The characterisation of the pathophysiology of MSD, MPSVI and PD has helped to identify disease mechanisms and markers and determine whether the treatment of secondary effects due to substrate accumulation can help / revert patient symptoms.
- The identification of tissue-pathology markers as determinants of Pompe disease stage have been crucial to determine the success of therapeutic intervention. These studies have potentially led to the identification of factors that modify the onset and clinical course of Pompe disease in patients with residual acid alpha-glucosidase activity, as well as to the identification of biomarkers that can be used to monitor the stage of disease and the effect of therapy. The findings are expected to be very relevant for the design of measures that can be taken to improve the natural course of Pompe disease and the outcome of therapeutic interventions, such as enzyme replacement therapy, chaperone based therapy and gene therapy.
- The characterisation of the natural history of a subset of LSDs will lead to a better insight in the natural course of rare LSDs and the response to therapy of patients with different ages and levels of disease severity. This information is important for the optimal timing and dosing of expensive new therapies like enzyme replacement therapy and for the development of treatment start- and stop criteria. Furthermore, in-depth knowledge has been obtained on the therapeutic effects on specific tissues, such as skeletal muscle and bone, the use of biomarkers in monitoring the effects of treatment, and the role of antibody formation and inflammatory parameters. Finally, the follow-up protocols and disease severity scales for Pompe disease and the MPSes can serve as a framework for studying other, very rare LSDs, such as fucosidosis, Farber disease and Niemann-Pick disease type A / B.
- The experimental results obtained have added information on the use of enzyme enhancement as an alternative therapy for Pompe disease and on the possible combined use of pharmacological chaperones and enzyme replacement.
Determining the safety and specificity of the putative therapeutic effects of iminosugars and the morpholino compounds as substrate-reducing drugs for the treatment of glycosphingolipidoses are of key importance in the long-term safety and benefit of this therapeutic modality; although work is still in progress towards the obtainment of an authentic viable model of Gaucher disease in the experimental mouse, the experimental results obtained thus far have been critical to a deeper understanding of the mechanism of action of these agents. The results have been informative on the specificity of the substrate-reducing drugs and their long-term therapeutic effect at their presumed biochemical site of action and have given confidence as to their safety and applicability in the other target lysosomal diseases for which their use has been proposed e.g. Fabry disease, Niemann Pick disease type C affecting the liver, GM1 gangliosidosis and the secondary glycosphingolipid storage disorders in MPSIII and related glycosaminoglycan storage diseases.

- The experimental results obtained on gene therapy have provided proof-of-principle that gene therapy may represent a new therapy for MSD or overcome some of the limitations of enzyme replacement for GD, PD and MPS VI.

Main dissemination activities and exploitation of results

The partners of the EUCLYD consortium have given particular attention towards the dissemination of the results of the project throughout these three years by presenting their data in international conferences, workshops and meetings and by publishing their results in high impact, peer-reviewed international journals (see templates A1 and A2 for more details).

The EUCLYD website has been developed and is online (www.euclyd.eu) as planned, which has favoured the dissemination of the project to the scientific community, patient groups, industry, biotechnology, and training institutions, and has facilitated the inter-communication among partners as well as the acceleration in work progression.

To this end, the site consists of a public area and a private area that is password protected (username: partners; password: europa3).

In the public area, which is easily accessible for any internet user, a first section (What is EUCLYD?) provides general information on the focus of the project, the diseases of interest to the consortium, and the aims and impact of the project in terms of European Union (EU) scientific and societal objectives.

A more specific description of the project work packages and their objectives is also provided together with a general overview of each partner making up the consortium, the role in the project, the key personnel and publications acknowledging EUCLYD. Links to other websites, relevant events and participation to meetings and symposia is also available. Finally, a separate section (Internal Use) with sensitive data (such as the TA, the contract and terms, the reports submitted to the EU, download to single publications) is restricted to the partners of the consortium and is password protected.

The EUCLYD management team has also given particular attention to keep the website up to date with the consortium annual meetings, including the PPTs presented by each partner and minutes of each meeting, and with the publications acknowledging EUCLYD and EU funding. The PPTs, minutes of the meetings and full articles of EUCLYD publications are again restricted to the partners and the EU and therefore are password protected.

As planned, the partners have been able to exploit the results of the project in their local research programmes, and have been successful in seeking additional major funding from national research agencies, foundations and industries to further extend the work carried out in the project towards the experimental characterisation of the most interesting findings.

Concerning the exploitation of results and contacts with industry, the consortium has continued to strengthen its contacts with the two industries (Actelion and Amicus) manufacturing Miglustat and similar compounds to develop new therapies using these drugs and to identify new indications for drugs already available in the treatment of LSDs. The partners involved in the treatment of patients (Prof. Andria, Prof. Beck, Prof. Cox, Prof. Reuser) have continued to involve the industries cited above in discussing preliminary data and implications of results in order to optimise the transfer of results from ‘bench to bedside’. In
In particular, UMC, LUND and UCAM now have a secure collaboration and we are confident that several further publications acknowledging EUCLYD will result within the next year at the latest (see for example, Herzog et al., in preparation in section 1.9 of periodic report month 19-36).

Unfortunately, work has been complicated by the worldwide total cessation of imiglucerase manufacture from June 2009. Since October 2010, supplies have been restored partially, but many patients have required adjustment to different enzyme preparations (VPRIV and taliglucerase) and additional patients have been started on miglustat (see WP4, M4.7 in periodic report month 19-36 for major details).

List of websites: www.euclyd.eu

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**Subjects**

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