Turning Science into a Cure

Sanfilippo Syndrome:

Current knowledge and perspectives for therapies

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I.1 Mucopolysaccharidoses

More than forty diseases are caused by lysosomal dysfunction, including Tay-Sachs, Gaucher, and Fabry disease, some leucodystrophies, and the Mucopolysaccharidoses. These so-called lysosomal storage diseases are caused by a missing or dysfunctional digestive protein, leading to the subsequent accumulation of substrates in the cell, resulting in very severe cellular and organ dysfunction.

Lysosomes are intracellular organelles derived from the Golgi apparatus by budding. They contain numerous digestive enzymes, mainly acid hydrolases, and are the site of macromolecules degradation, in a permanent recycling process. Cellular and extracellular macromolecules are constantly destroyed and replaced by new synthesised ones. The lysosomes have an acidic milieu (pH 4,8). A single phospholipid membrane stabilises their internal composition and maintains the low pH by pumping protons (H+) from the cytosol. The digestive enzymes need the acidic environment to function correctly a feature which limits the damage to the cell in case of disruption of the lysosomal membrane . All these enzymes are produced in a particular compartment of the cell, the endoplasmic reticulum, and further transported and processed in the Golgi apparatus. Most of these enzymes are targeted to the lysosome by a phosphorylation signal. This discovery of this process is the basis of therapeutic approaches aimed at providing the missing enzyme from an external source.

Mucopolysaccharides or glycosaminoglycans (GAGs) are constantly recycled macromolecules of essential importance for normal cell function. In mucopolysaccharidosis (MPS), a deficiency in one of the lysosomal enzyme that participates in the stepwise degradation of GAGs leads to their accumulation, and results in severe cell dysfunction. MPS form a complex group of genetic diseases differing by their genetic origin, biochemical and physiological disturbances and clinical manifestations. Depending of the mutated enzyme, the catabolism of one or several types of GAGs will be blocked, some enzymes being involved in the degradation pathway of multiple GAG species. Eleven known enzyme deficiencies are responsible for 7 MPS.

MPS I (□-L-iduronidase deficiency), MPS II (iduronidate sulfatase deficiency) and MPS VII (□-glucuronidase deficiency) include CNS symptoms in their severe forms, together with very severe somatic involvement. In addition to heparan sulphate catabolism blockade, this three enzyme deficiencies lead to the blockade of other GAGs catabolism, namely dermatan sulfate in the case of MPS I and II, and dermatan sulphate and chondroitin sulfates in the case of MPS VII.

In addition to the mental retardation, the somatic symptoms include organomegaly, dysostosis multiplex, and other organ dysfunctions.

The biochemical diagnosis is usually done by measurements of urinary GAGs and/or enzyme assays on patient leucocytes and fibroblasts.

I.2 Sanfilippo Syndrome

Sanfilippo syndrome or MPS III can result from four different enzyme deficiencies. These four enzymes are involved in the lysosomal catabolism of heparan sulphate glycosaminoglycans which are heparin-related macromolecules consisting of a chain of carbohydrate monomeric residues, some of which are sulphated. They are involved in growth factors signalling pathways, but their functions are not yet totally elucidated. The eight enzyme involved in the stepwise degradation of heparan sulphate are believed to function in cooperation in an enzymatic complex inside the lysosome, the product of one enzyme being passed to the next one (Freeman and Hopwood, 1992). Although diverse at the genetic and biochemical levels, the clinical manifestations are very similar in the four forms of the disease.

The cause of Sanfilippo type A is a deficiency in Heparan-N-sulfatase, also named sulfamidase. In Sanfilippo type B, the □- N-acetylglucosaminidase is deficient. These two forms of the disease are the most common. Type C and D are very rare and correspond respectively to a deficiency in □-glucosaminide acetyltransferase and N-acetyl glucosamine 6-sulfatase. These four enzyme deficiencies are the consequence of genetic mutations occurring on their respective genes, and the four diseases are transmitted on an autosomal recessive mode of inheritance.

I.2.1 Incidence

Two reference studies have been published recently on the incidence of MPS in Western Australia (Nelson et al., 2003) and Germany (Baehner et al., 2005) respectively. The Australian study reported the incidence of MPS between 1969 and 1996 in the western Australian population of approximately 1.9 million individuals. Sanfilippo cases were found to be exactly half of all MPS cases (eleven on 22). Three of the eleven cases were diagnosed prenatally (one MPS IIIA and two MPS IIIB). Among the 8 live born cases diagnosed as MPS III, four were IIIA, one IIIB and one IIIC. No Sanfilippo type D was diagnosed during this extended period of twenty seven years in this population. According to this study, the total incidence of MPS is 3,43 per 100 000 live births. The total incidence of Sanfilippo syndrome is 1,71 per 100 000.

The German study encompasses a period between 1980 and 1995. The total incidence of all MPS was 3.53 per 100 000 births. Considering Sanfilippo syndrome, the total incidence is 1,57 per 100 000 births. Approximately 2/3 of the cases (149/211) were Sanfilippo type A, 49/211 type B and 13/211 type C. No type D was diagnosed during this period. Poorthuis et al. reported an incidence of 1.89 per 100 000 live births in the Netherland (Poorthuis et al., 1999). No similar studies are currently available concerning the French population.

I.2.2. Diagnostic techniques and diagnostic reference center in France.

Because of the mild somatic signs, and given that the symptoms of Sanfilippo syndrome are common in the general population, at least at the onset of the disease, the diagnosis is often a delayed after the onset of symptoms.

Generally, MPS disorders are diagnosed by their main biochemical features, i.e. accumulation of GAGs, detectable in urinary samples, and enzyme deficiencies deficiencies assays, most of the time measured in patient measured in leucocytes. A considerable body of body of work has been carried out in John Hopwood's group in Adelaide, Australia in this domain (Fuller et al., 2004). A general effort was aimed at characterising the accumulated compounds in each enzyme deficiency. New technological developments are currently applied to refine the detection and identification of GAGs metabolites in thein patient urine and fibroblasts. In the specific case of Sanfilippo syndrome type A, a well documented very recent study in MPS IIIA versus normal mouse tissues (brain, liver and spleen) validates the use of "electrospray ionization tandem mass spectrometry" for the detection of an heparan sulphate-derived disaccharide (a monosulfated disaccharide fragment) as a marker that accumulates abnormally in this disease (King et al., 2006). Although this technique has not been validated on patient sensitivity has not been yet validated on patients urine samples yet, theis heparan sulphate metabolite maymay probably be considered as a useful candidate surrogate marker useful that will allow to monitor the effects of treatments in the human disease. in the human disease that will allow to monitor effects of the treatment in human disease, in the case of enzyme replacement therapy for example. Since enzyme replacement in the case of Sanfilippo syndrome will have to be performed in the CNS the markers measurements will be performed s will have to be performed in the cerebrospinal fluid.

On the enzyme side, the diagnosis of Sanfilippo disease is generally made by measurement of each particular enzyme activity. In France, the reference center for these diagnosis is the "laboratoire de biochimie pédiatrique, Hopital Debrousse, Hospices Ccivils de Lyon", directed by Dr Irene Maire. In the case of Sanfilippo type A, Sulfamidase activity is measured in this center and in most of the labs in the world using artificial fluorogenic susbtrates, (Karpova et al., 1996). Although this technique is relatively simple and allows today the routine

diagnosis, there is definitely a need for more sensitive assays enabling the detection of very low enzyme quantities. John Hopwood's group has developed two specific assays for sulfamidase quantification and activity measurement respectively. A highly sensitive assay using radiolabelled oligosasaccharides, a natural substrate of the enzyme, allows the detection of very low residual activity in patients cultured skin fibroblasts (Hopwood and Elliott, 1982b). Similar assays are available in the cases of Sanfilippo B, C and D (Elliott and Hopwood, 1984; Hopwood and Elliott, 1981; Hopwood and Elliott, 1982a) respectively. A "sandwich Enzyme Linked Immunosorbant Assay (ELISA)" also developed in this laboratory, allows the immunoquantification of the total amount of enzyme (including both both active and inactive enzyme) produced by the cells. When combined, these two techniques allow an accurate prediction of the disease severity (Perkins et al., 2001). Moreover, the combination of this three measurement techniques should allow a correct evaluation of the outcome of a pharmacological treatment allowing the production of little amounts of enzyme, for example in the case of readthrough processes strategies (See below).

When the risk of MPS III is known, prenatal diagnosis is available using cultured cells from chorionic villi samples at early stages of pregnancy, i.e. 9-10 weeks gestation, or cultured cells from 15-16 weeks amniocentesis, (Hopwood, 2005). Newborn screening for lysosomal storage disease in generally under study/evaluation (Meikle et al., 2006).

I.2.3. Genetic basis of Sanfilippo syndrome

The genetic diagnosis of this disease is not yet routinely available worldwide, although it is in France, in the reference center of Lyon (See above). Considerable development has been made in this domain, leading to a more detailed knowledge of the genetic mutations underlying the respective enzymatic deficiency, and the genotype/phenotype relation in the different diseases. With the exception of MPS IIIC, for which the gene is localised but not yet identified, genetic diagnosis and further studies of specific mutations are now possible, and new data including description of new mutations and their associated phenotypes are accumulating.

In the case of MPS IIIA, the gene encoding sulfamidase is relatively small (11 kb) includes 8 exons, and is localized on chromosome 17 (17q25.3). This gene was characterised in 1996 (Karageorgos et al., 1996) and encodes a protein of 502 amino acids. For information, the sufamidase protein is also named N sulfoglucosamine sulfohydrolase (SGSH) and the gene and protein are referenced under theseese initials on the NCBI site,s with the accession numbers of NM 000199.2 and NP 000190 respectively.

The vast majority of the reported genetic defects are missense mutations. Deletions and insertions also occur. Point mutations resulting in a premature stop codon have been found in 11% of Sanfilippo type A cases in the Lyon diagnostic center (Irene Maire, personal communication). More than 60 mutations have been reported, with a great discrepancy in their

relative frequency. Founder effects can probably explain per example a high frequency of 1091delC in Spain, and R245H in Dutch and German populations. Patients described with attenuated forms of Sanfilippo syndrome type A presented homozygous mutation R206P (Di Natale et al., 2003; Esposito et al., 2000), heterozygous mutations S347F/D444G (Miyazaki et al., 2002), and E369K/P128L (Di Natale et al., 2003). The R206P mutation affects a non conserved amino acid residue between the murine and human enzyme (Weber et al., 2001). This fact could explain a milder alteration of the enzyme function. Several polymorphisms have also been identified in this gene. Among them, R456H has a high frequency in the the Australian population, and was reported to be associated to the homozygous R206P mutations mentioned above in the patient with an attenuated form, although there is no other clue on the potential role of this polymorphism in the context of this mutation. A recent case report mentioned a young patient with hepatomegaly carrying heterozygous mutations (Bekri et al., 2005). A premature stop codon appeared on one allele with the mutation Y40X. The other allele contained two distinct mutations, E300V and Q307P.

I.2.4 Clinical manifestations

Sanfilippo syndrome is characterised by severe central nervous system (CNS) degeneration, with mild somatic symptoms. The main involvement of the central nervous system is unique to MPS III. The child appears normal until the onset of clinical symptoms, which usually occurs between 2 and 6 years, but may occur earlier or later. This signs include a marked hyperactivity with aggressive behaviour, delayed development, sleep disturbances, mental retardation and mild hepatomegaly (most of the time in the young patient). Contrary to others MPS, coarse facial features are not prominent in this disease, and some patients have normal features. The skeletal involvement is mild too, the stature is normal, with mild joint stiffness. Severe hearing loss is common. Seizures occur later on, but can be controlled by adequate medication. Early onset of puberty has been reported in MPS IIIA male patients (Tylki-Szymanska and Metera, 1995). Neurologic degeneration occurs between six and ten years of age, but progression to severe cortical atrophy occurs only in the late stages of the disease (Zafeiriou et al., 2001).

Very mild forms of Sanfilippo syndrome are believed to be undiagnosed, and few patients only have been described with a less severe form of the disease (Wraith et al., 1987). Severe and attenuated forms have been reported in the same family in the case of Sanfilippo B (Di Natale, 1991), showing that identical genotypes can underlie a broad clinical spectrum. There are adult patients with Sanfilippo A even if this form is reported to be more severe (Lindor et al., 1994). According to Dr Di Natale, (Gabrielli et al., 2005), only 10 cases of attenuated forms of Sanfilippo type A syndrome have been described with a specific genetic mutation identified only in two of

them. In this study, the authors describe a twenty year old patient presenting with a mild clinical phenotype characterized essentially by a moderate non-evolving mental retardation. The enzymatic activity of this patient was estimated to be about 8% of the normal activity. This study is to be related to the small amounts of enzyme already reported to lead to a reduction of storage and probably to an alleviation of the clinical symptoms (Hopwood and Morris, 1990).

I.2.5 Cellular dysfunction and neurodegeneration

The physiopathology of the disease is not well understood. If the primary event at the cellular level is indeed the loss of enzymatic activity leading to the accumulation of undegraded substrates within lysosomes, the consequences are very diverse. Lysosomal extension occurs with abnormal structures observable by electron microscopy. Surprisingly, the levels of other lysosomal enzyme are increased, the phenomenon remaining unexplained.

The links between heparan sulfate lysosomal storage and neuronal dysfunction/degeneration is not yet elucidated. The accumulation of Heparan sulphate species is believed to affect signalling by members of the fibroblast growth factor (bFGF) family (Pellegrini, 2001). These factors have important roles in neural cell physiology (Reuss and von Bohlen und Halbach, 2003). In the Sanfilippo type B mouse model, a marked attenuation of neuronal and glial plasticity has been reported (Li et al., 2002). Dr Neufeld's group has also described a marked GAG accumulation in microglial cells, the resident monocyte derived cells (macrophages) in the brain (Ohmi et al., 2003). These cells are also found in an activated fate. These observations are in favour of a profound dysfunction of glia - neuron cross talk. Neuronal cells indeed undergo storage too, and a progressive dysfunction ultimately leading to neurodegeneration. The neuronal degeneration itself is probably a late phenomenon, as cortical atrophy is not observable before the late stages in patients by magnetic resonnance imagery (MRI) (Zafeiriou et al., 2001). There might be some discrepancies in the time course of cerebral lesions depending on the neuroanatomical structure, although this aspect has not been explored in patient brains due to the lack of autopsy studies. White matter loss is already detectable at advanced stages, probably reflecting the loss of nerve fibres. This late phenomenon are obviously irreversible, however, the progressive course of the disease provides an opportunity for therapeutic interventions once they will be available.

An important process of secondary gangliosides accumulation has been described in different MPS mice models (McGlynn et al., 2004; Siegel and Walkley, 1994). Gangliosidoses storage occurs in another group of lysosomal storage diseases, the glycosphyngolipidoses, in which the missing enzyme controls the degradation of lipid compounds or glycosphyngolipids (GSL). The link between heparan sulphate accumulation and subsequent ganglioside accumulation is not yet understood. Nevertheless, this observation, led to the hypothesis that

gangliosides storage may also have a part in the MPS nervous system pathogenesis. A small molecule named Zavesca, and produced today by the company Actelion Parmaceutical is currently available on the market and used as a routine medication in Gaucher disease (Elstein et al., 2004), one particular GSL storage disease, in which there is a primary accumulation of gangliosides. Gaucher disease is a member of a family of inherited disorders called sphingolipidoses that among others includes Tay-Sachs and Sandhoff diseases. It is caused by the accumulation of glucosylceramide (glucocerebroside) due to deficient activity of the enzyme glucosylceramide-beta-glucosidase (glucocerebrosidase). The iminosugar molecule (Zaveska or miglustat) is an inhibitor of the substrate (GSL) synthesis, such limiting its flux to the diseased lysosome. Important clinical knowledge has been now accumulated on its long term use including its use in very young patients in the case of Tay Sachs disease (Bembi et al., 2006). This molecule is currently under examination in a long term clinical trial aimed at studying it's effect on Nieman Pick C disease, (Mark Patterson, Columbia University, New York), another lysosomal storage disease involving the brain, in which the ganglioside accumulation is also a secondary process (Zervas et al., 2001). A clinical trial is in preparation in France (Dr Guffon, Lyon) in Sanfilippo syndrome, in order to provide a rationale for the use of this drug in that particular disease. Theoretically, the alleviation of gangliosides accumulation should slow the time course of the disease, as it is difficult to imagine that their secondary accumulation have no effect at all on the neuronal dysfunction. It is indeed demonstrated that ganglioside accumulation gives rise to ectopic dendritic groth on neuronal cells (Walkley, 1995; Walkley et al., 2000). Due to the weak inhibition potential of ganglioside synthesis of this molecule, a therapeutic effect should be observable only on the long term (the Columbia University clinical trial has been designed for two years).

Importantly, ganglioside accumulation (GM2 and GM3) has been reported in a post mortem study of two human Sanfilippo III D brains (Jones et al., 1997).

The disappearance of gangliosides accumulation in the brain has also been shown to be a surrogate marker of neuropathological improvement in a gene therapy strategy carried out in MPS IIIA mice (Cressant et al., 2004). This observation is also a demonstration of such lesions reversibility.

I.2.6 Animal models

In vivo studies and the subsequent development of new therapeutic approaches are dependant on the availability of animal models of human disease. Research on sanfilippo syndrome has probably suffered because of the lack of such models over the time. Relatively recently, a mouse model of Sanfilippo type A was discovered (Bhaumik et al., 1999), carrying a missense mutation on the sulfamidase gene (Bhattacharyya et al., 2001). Although this mice display some residual enzymatic activity, they present clinical symptoms close to the human manifestation of the disease, and their nervous tissue is an excellent study material for elucidating the pathophysiology of heparan sulphate accumulation and its consequences. They are an excellent resource for the study of enzyme replacement and gene therapy strategies for example. A Sanfilippo A dog model is available too in the form of a colony in New Zealand, and displays features of the human disease (Jolly et al., 2000). In this case, the mutation of the sulfamidase gene is an insertion (708-709insC) (Yogalingam et al., 2002). For Sanfilippo type B, a knock out mouse has been created by Liz Neufeld's group, such allowing numerous research studies on this particular form of the disease (Li et al., 1999). A dog model has also been discovered (Ellinwood et al., 2003) and is currently used for gene therapy experiments at the Iowa State University. A caprine model of Sanfilippo type D was the first animal model to be described for Sanfilippo disease (Thompson et al., 1992). Although type D is the rarest type of Sanfilippo syndrome, studies carried out on this model were useful for the understanding of Sanfilippo syndrome in general. Enzyme replacement therapy has been studied in this goat model (Downs-Kelly et al., 2000). Interestingly, the genetic defect in the N-acethylglucosamine-6- sulfatase gene has been early identified. It consists of a single base substitution creating a stop codon in the 5' region of the coding sequence (Cavanagh et al., 1995). This Sanfilippo type D goat model could be useful for the investigation of the safety, central nervous system availability, and therapeutic efficiency of small molecules with premature stop codon readthrough potential (see below).

II. GENE AND CELL THERAPY FOR SANFILIPPO SYNDROME

II.1 Cell therapy

II.1.1 Hematopoietic stem cell transplantation

Bone marrow transplantation (BMT) considerably improves the condition of MPS I patients (Hurler syndrome) and has a therapeutic effect on neurologic symptoms in this disease. Yet, the benefit of BMT in lysosomal disorders is very variable from one disease to the other. Microglial cells are resident macrophages of the nervous tissue which undergo a slow renewal throughout life. These cells derive from the monocyte population of the blood, and are able to enter the central nervous system by transcytosis through the blood capillaries. This mechanism is thought to be at the basis of the therapeutic effect of BMT in some of the lysosomal storage disease with central nervous system involvement. For example, in mannosidosis, the enzyme has been shown to be secreted by the microglia and recaptured by adjacent neuronal cells in the mutant mouse model of the disease. This process may differ in the different forms of MPS III, in which no therapeutic effect have been obtained (Sivakumur and Wraith, 1999). In this study, early transplantation in one asymptomatic child have not led to reversion of disease manifestations (Sanfilippo A) despite successful engraftment. It is also possible that the level of microglial turn-over is not sufficient in that cases to provide enough enzyme for an alleviation of neural cell dysfunction.

Allogeneic cord blood transplantation has been proposed in this syndrome by Dr Kurzberg at Duke University (North Carolina, USA). The procedure results in a high morbidity and mortality, and there is no current evidence that cord blood derived cells are more potent in gaining entrance into the brain and is more effective than other sources of donor cells.

II.1.2 Intracerebral cell transplantation

In an attempt to overcome the blood brain barrier, intracerebral cell transplant have been carried out with human neural stem cells in a murine model of MPS VII or Sly disease (Snyder et al., 1995). This approach is aimed at providing an internal source of normal enzyme inside the brain, but poses first the problem of the cells source. Human cord blood cells have been grafted in a Sanfilippo B mouse model brain, showing a certain migration potential, and an ability to survive more than seven months. Interestingly, liver GAGs accumulation was diminished in these experiments, showing that the enzyme produced in the brain may gain the periphery and have a therapeutic effect on somatic symptoms (Garbuzova-Davis et al., 2005). In general, such

approaches need more investigation, mainly on the mortality of grafted cells, their potential tumorogenicity, and the immune response directed against cells producing an unknown enzyme. The immune reaction can not be studied in mice, and has to be examined in dog models. To overcome this problem, it has been proposed to sort human fetal central nervous system stem cells and expand them in vitro prior to intracerebral grafting (Tamaki et al., 2002).

II.2 Gene Therapy

II.2.1 Gene transfer with autologous stem cell transplantation

Hematopoietic stem cell transplantation with autologous cells preceded by *ex vivo* transduction was explored in MPS VII mouse model (Marechal et al., 1993). This study showed a widespread correction of systemic manifestations of the disease. Enzyme activity was also detected in brain tissue. More recent studies in the Sanfilippo type B mouse model demonstrated some therapeutic effect of autologous stem cell transplant after ex vivo gene transfer with retroviral vectors (Zheng et al., 2004) and lentiviral vectors (Di Natale et al., 2005). Brain tissue lesions were in part corrected in mice expressing very high levels of □-N-acetylglucosaminidase, and microglia with engorged lysosomes almost completely disappeared. To date there is no study in Sanfilippo type A mice model with this particular therapeutic approaches.

Several gene therapy strategies were aimed at obtaining an internal continuous source of secreted protein. *Ex vivo* transduction of autologous mitotic cells using retroviral vectors and their subsequent grafting were performed in mice and dog models of MPS. This general approach of systemic delivery was performed using skin fibroblasts. First tried with allogeneic skin fibroblasts in MPS II, this approach led to relative biochemical improvement (Dean et al., 1976), but did not show any effet on the course of the disease. Like bone marrow cells, skin fibroblasts can be obtained from biopsies and genetically modified using retroviral vectors, thus offering the possibility of corrected cells autologous transplantation. An original approach consisted in implanting this modified fibroblasts in the form of neoorgans in the peritoneal cavity of adult mice (Moullier et al., 1993) and dogs (Moullier et al., 1995). These neoorgans consisted of collagen, and polytetrafluoroethylene fibers containing the modified cells with basic fibroblast growth factor (bFGF). They persisted for months, leading to a correction of liver and spleen pathology, and with very little enzyme activity in the brain.

The question here is the assessment of the therapeutic interest of high doses of secreted enzyme in peripheral tissues for the alleviation of central nervous system disease. In the case of hematopoietic stem cell transplantation, blood borne derived cells (monocytes) are believed to

gain access to the brain were they renew the microglial cell pool, this renewel being at the basis of the enzyme availability in the nervous system.

II.2.2 Direct gene transfer into peripheral organs

Variations of such strategies of systemic enzyme delivery by autologous cell genetic modification were explored by direct injection of the gene vectors into muscle or liver in animal models of MPS. Both of these tissues contain mainly post mitotic cells and gene transfer would only be possible in these cases by using vectors able to tranduce such cells. It is the case of adeno associated viral (AAV) vectors. This approach was extensively explored in the context of hemophilias, and has led to clinical trials in hemophilia patients (Manno et al., 2003). A low level of intracerebral enzyme activity was also detected in a recent study after intrahepatic transduction with AAV vectors in MPS VII mouse model (Sferra et al., 2004).

II.2.3 Direct intracerebral gene transfer

Lysosomal storage diseases have long been considered as a model of choice for gene therapy strategies. Among them, Sanfilippo syndrome poses the general problem of gene transfer to the central nervous system. Viral vectors, i.e. recombinant replication deficient viruses, are engineered to carry the normal form of the mutated lysosomal enzyme. The gene transfer vectors known to date do not cross the blood brain barrier when administered systemically, and direct intracerebral administration is considered the approach of choice to overcome this barrier. A number of considerable advances have been made since ten years in the identifications and characterisation of adequate vectors meeting the specific constraints for an efficient gene transfer into the brain. Among the vectors currently in use in gene therapy strategies, adeno associated vectors (AAV) seem to display particular properties, such as a relatively good diffusion in the brain parenchyma, a certain degree of neurotropism (varying from one serotype to the other), thus leading to an efficient transduction of neurons. The native viruses from which the vectors are derived are single strand DNA parvoviruses. Their life cycle is dependant of the presence of other viruses, adenovires or herpesviruses. No human disease has been currently identified as a consequence of AAV infection. The vector genome is deprived of viral gene sequences. These vectors are believed to trigger very limited immune or inflammatory reaction, if any, after a first administration. Conversely to other vector types, such processes have not been observed in the case intracerebral injection of AAV vectors. Preclinical studies data indicate a very stable transgene expression for years after vector administration to dogs. Clinical trials have been currently performed by intramuscular injection in hemophilia patients (Manno et al., 2003).

Viral vector preparation is administered directly into the brain by stereotactically guided injection. This technique allows targeting selective neuro-anatomical sites for controlled the vector

delivery. Although the spread of AAV vector in the brain parenchyma is limited, enzyme secretion, diffusion and capture from adjacent cells whould allow a high degree of correction throughout brain tissue. Transduced cells in the brain whould express and deliver the enzyme continuously, thus constituting an intracerebral permanent source of enzyme production.

In the United States, three ungoing clinical trials are based on intracerebral gene transfer by AAV vectors. One of them concern a lysosomal disease, Late Infantile Neuronal Ceroid lipofuscinosis (LINCL), and is carried out in New York under the leadership of Dr Ron Crystal, Cornell University. Ten patients have already been treated in 2005. Patients were divided in two groups, four patients with the severe form of the disease, and six patients with the moderate form of the disease (Crystal et al., 2004; Passini et al., 2006).

For Sanfilippo disease, gene therapy using recombinant AAV vectors directly administered into the brain has been investigated in the mouse model of Sanfilippo B (Cressant et al., 2004; Fu et al., 2002). Muenzer's group showed the efficiency of AAV gene transfer in alleviating the intracerebral lesions, and tested comparatively two different promoters. JM Heard and coll's study addressed in detail the issue of widespread delivery of the enzyme throughout the brain, together with a behavioural study showing improvement in mice scores measuring anxiety and activity levels. Moreover, two different AAV vector serotypes are studied, namely AAV2 and AAV5. The NAGLU activity was higher and its distribution broader with AAV5 – NAGLU vectors. An important point is that the accumulation of gangliosides present before treatment was reversed, showing that this secondary accumulation should be considered as a surrogate marker for the evaluation of the alleviation of CNS lesions in animal studies. This Pasteur Institute group is currently implementing these studies by investigations in the dog model of the disease, in collaboration with Dr Matthew Ellinwood in Iowa State University. Studies in the dog model allow considering the immune reaction against the newly secreted protein, and the design and evaluation of immunosuppression regimen transposable to patients in a clinical trial protocol. A clinical trial is under preparation, and will be performed in France. This type of study is not currently available in Sanfilippo type A mouse or dog model. Ongoing studies are performed in John Hopwood's group (Adelaide, Australia).

III. READ THROUGH OF PREMATURE STOP CODON: A NEW THERAPEUTIC APPROACH FOR A SUBSET OF MPS III PATIENTS WITH APPROPRIATE MUTATIONS

Mutations that change a codon in such a way that they no longer specify an amino acid but are recognized as a translation stop are called nonsense mutations. They result in the truncation or absence of a protein product, and are associated with a vast panel of genetic disease. Three distinct stop codon sequences are used in genes: UAA (ochre), UAG (amber), and UGA (opal). Each have different fidelity for the process of sequence termination. Generally, UGA is considered as having the highest natural readthrough potential (Lovett et al., 1991). UAG shows an intermediate fidelity, and UAA is known as the high fidelity stop codon, having the smallest natural readthrough potential. The surrounding mRNA sequences are considered to have an important influence on the fidelity of stop codons (Bonetti et al., 1995; Lovett et al., 1991; Mottagui-Tabar et al., 1998).

Ten years ago, gentamicin and other aminoglycoside antibiotics were shown to “suppress” stop codon arrest by allowing the ribosome to read through these codons and insert an amino acid.

We will summarise here the research data obtained with gentamicin on patients cells and animal models, as well as a few pilot clinical studies that have been carried out on patients with premature stop codon mutations in various genetic diseases.

III.1 Research studies on gentamicin and other amynoglicosides as agents for readthrough of premature stop codon

A structural study by Yoshizawa et al. showed that gentamicin exerts its antibiotic action by targeting the 30S ribosomal subunit, where it interferes with the initiation complex of protein formation (Yoshizawa et al., 1998). Earlier studies had suggested that the aminoglycoside antimicrobial effect was due to a specific interference with the process of proofreading that allows the discrimination against mismatched amino acyl-transfer RNA from being incorporated into the growing polypeptide chain (Ruusala et al., 1984).

Gentamicin is assumed to promote premature stop codon suppression in mammalian cells by a related binding event (Burke et al., 1985; Martin et al., 1989). Aminoglycoside antibiotics suppress stop codons with different efficiencies (UGA > UAG > UAA), although the the ability to read past these codons is further dependant upon the local sequence context (Bonetti et al., 1995; Manuvakhova et al., 2000; Mottagui-Tabar et al., 1998). Studies have shown that glutamine

and tryptophan are the two most common amino acid insertions for stop codon readthrough (Harrell et al., 2002; Lovett et al., 1991). In general, suppression rates tend to be low, and side effects of long term amynoglicoside use can be severe. This created a need for the discovery of more potent and less toxic compounds. The main study demonstrating high dose gentamicin efficiency in restoration of dystrophin in mdx mice was published by Lee Sweeney 's group in 1999 (Barton-Davis et al., 1999). This was the first demonstration of a suppression of a premature stop codon by aminoglicosides an an animal model of human genetic disease. The mdx mouse model of Duchenne Muscular Dystrophy (DMD) carries a premature UAA codon in the 23rd exon of the dystrophin gene. In most of the treated mice, the level of rescued dystrophin was 10-20% of the normal, which is compatible with an alleviation of the symptoms. Indeed, the authors were able to show that treated mice displayed a significant functional protection against contraction-induced injury.

In another work, the effect of gentamicin treatment was studied on human DMD mutations (Bidou et al., 2004) in an elegant experimental system of muscle transfection in mice. This study also confirms that human premature stop mutations could be misread under gentamicin treatment, leading to restoration of dystrophin expression. Yet this study documented very low levels of readtrough.

Studies with aminoglycoside readthrough in Hurler disease could be of particular interest for other lysososmal diseases. It is indeed a shared characteristic for most of this disorders that small amount of restored enzyme is enough to reverse the disease process or at least to result in a milder form of the disease.

In the case of Sanfilippo syndrome, it is believed that mild forms, if they exist, are not diagnosed, as the vast majority of the cases known to date are severe. In that disease, central nervous system involvement is the major hallmark of the disorder, and here again, a main issue for a candidate therapeutic compound would be its availability in the nervous tissue.

In the most recent study by John Hopwood's group targeting the stop codon mutations of MPS I patients, the authors have first characterized such L iduronidase (IDUA) mutations. Premature stop codon mutations are very common in this disease, accounting for up to 70% of MPS I disease alleles in some populations. In the Adelaide cohort, 90 % of the patients had a stop codon mutation in at least one mutant allele. The authors have then studied in detail the potential readthrough of this particular mutations (Hein et al., 2004). They have investigated the outcome of each of the possible stop codon at the same locations (Q70X and W402X) both in terms of natural and gentamicin-induced stop codon readthrough. The study was carried out in a CHO-K1 cell expression system, this mutant CHO cell line being designed for specific detection of protein resulting from transfected constructs. The introduction of all possible stop codon at these two sites led to an increased mRNA instability and in one case to a complete loss of mRNA. The authors

demonstrated a very strong context effect, enabling them to compare the same stop codons in different location on the same gene. In all but one cases examined, gentamicin treatment led to a low increase in enzyme activity, without changing the amount of mRNA. In a previous study, the authors have described that gentamicin treatment of MPS I patients fibroblasts cells could lead to an IDUA activity corresponding to 2.8% of normal (Keeling et al., 2001). This study interestingly showed that low level of IDUA stop codon readthrough could reduce glycosaminoglycan (GAG) storage and vacuolation in patients' fibroblasts. Although this study can not be extrapolated to other MPS genes containing stop codon mutation, it illustrates that a very low level of enzyme correctly processed inside the cell could have a certain efficiency in reversing the cellular hallmarks of the disease.

III.2 Clinical trials using gentamicin

A number of short term clinical trials have been carried out to investigate the outcome and clinical interest of gentamicin use in subsets of patients with different genetic diseases, which carry premature stop codon mutations. In general, suppression rates tend to be low, and together with the long term aminoglycoside use is associated with intractable side effects. This clearly points out to a need for the discovery of more potent and less toxic compounds.

III.2.1 Hemophilia

A recently published report describes the results of a clinical trial in five hemophilia patients, which was carried out in Canada (James et al., 2005). These severe haemophiliac patients, 3 with haemophilia A and two with haemophilia B, with known non sense mutations, were treated 3 consecutive days with gentamicin at a dose of 7mg/kg intravenously every 24 hours. For this very short treatment time, none of the patients showed any renal or ototoxicity or other adverse event linked to the treatment protocol.

Small amounts of clotting factors were detected in two patients. It must be stressed that due to the well known gentamicin toxicity and to the status of patients, the protocol regimen was only of 3 days. Although unsatisfying, the existing response of patients in such clinical trials, is in favour of studies with alternative drugs that may be capable of higher readthrough efficiencies (PTC 124, see below), provided that toxicity issues could be addressed.

III.2.2 Duchenne Muscular Dystrophy (DMD)

In the first trial reported in the literature, four DMD or Becker (milder form of the disease) patients were treated with gentamicin at a regimen of 7.5mg/kg/day for 2 weeks (Wagner et al., 2001). The full length dystrophin protein could not be detected in these patients. No adverse event was reported in this trial.

In a second study carried out in Naples, Italy, and with a different protocol (two six day cycles with an interval of seven weeks), dystrophin positive fibers were seen in three out of four patients, who had the low fidelity stop codon UGA. In one of this patients, dystrophin re-expression was qualified as dramatic. The fourth patient had a high fidelity UAA stop codon and did not show any expression of dystrophin (Politano et al., 2003).

III.2.3 Cystic Fibrosis (CF)

In 2001, a first study in five patients already gentamicin parenteral treatment showed that could produce small increases in CFTR (Cystic Fibrosis Transmembrane Receptor) CL conductance (Clancy et al., 2001). Although in this disorder too, the question remains open to how much CFTR activity is needed for a therapeutic purpose.

More recently, a double blind, placebo-controlled, cross-over trial (14 days treatment) was carried out in Israel (Wilschanski et al., 2003). Translational readthrough was again demonstrated together with a correction of the typical electrophysiological abnormalities caused by CFTR dysfunction.

All these results were very encouraging because they evidence for the possibility of readthrough of premature stop codon in diseased patients. Nevertheless, long term use of aminoglycoside antibiotics is associated with ototoxicity and nephrotoxicity, which may limit their utility in readthrough therapy.

III.2.4 Clinical trials using PTC 124

PTC 124 is a new compound discovered through the technology of high throughput screening developed by the biotechnology company PTC (for Post Transcriptional Control), based in New Jersey (See the website http://www.ptcbio.com/big/discovery1flash.html). The screening for molecules able to bypass a premature stop codon was developed on a luciferase activity assay. (The luciferase gene was mutated and contained one of the three stop codons at a premature location).

Orally available, this compound has no antibiotic activity and is presumed to interact with the ribosomal 18S RNA during translation.

To date, no scientific publication is available on this compound, although its effects have been investigated in a phase one clinical trial, and two Phase two clinical trials are currently undergoing, on Duchenne Muscular dystrophy (DMD) and Cystic Fibrosis (CF). The question of readthrough of the real stop codon is still open. In the phase one clinical trial, three plasma protein from treated healthy volunteers were shown to be properly translated, with no appearance of longer forms (Although such forms should be difficult to detect). The specificity to bypass only premature stop codons is assumed to be due to steric constraints not allowing the interaction of the compound at the end of the RNA sequence

General safety was assessed in the phase one clinical trials together with pharmacokinetics studies for the design of appropriate dosing regimen for phase two trials. Although little is known on the potential long term toxicity, no major side effect has been described to date in the healthy volunteers of this trials (14 days of treatment) or in patients currently under investigation in the Cystic Fibrosis Phase two trial. At very high doses, healthy volunteers have experienced side effects like nausea, headache and dizziness. This compound is also well tolerated in dogs for up to one year treatment at higher doses than the requirement for correct plasma concentration in humans. Interim results of the CF trial have been recently disclosed and do not indicate any safety problem (http://www.ptcbio.com/big/pr040406.html).

Obviously, if the therapeutic efficiency of PTC 124 is demonstrated in these two multicentric clinical trials, it would bring a great hope for the development of clinical trials for a subset of patients in lysosomal storage diseases.

In the particular case of Sanfilippo syndrome, and with the hypothesis that PTC 124 could have a therapeutic effect on patient fibroblasts, the question again would be of its central nervous system delivery. PTC 124 is available in the CNS in low quantities when delivered orally to rats (PTC, personal communication). Due to the primary CNS involment in the disease, more data are needed on this compound availability in the CNS after peripheral delivery. After a proof of concept would be available on patients' fibroblasts carrying premature stop codons, we would need to know if oral delivery of higher doses is possible in order to achieve therapeutic levels in the CNS, or if the compound has to be delivered directly inside the CNS by specific means (devices, intrathecal injection). These questions deserve further comprehensive animal studies.

IV. ENZYME REPLACEMENT THERAPY: PERSPECTIVE FOR SANFILIPPO TYPE A SYNDROME

IV.1 Rationale for enzyme therapy in Lysosomal Storage Disorders

The theoretical basis for enzyme replacement therapy (ERT) was provided by the discoverer of lysosome C. de Duve in 1964, who noted that molecules taken up intracellularly by an endocytic process from the extracellular milieu would appear in lysosomes (de Duve, 1964). Experimental support then came from Liz Neufeld's lab, demonstrating that the abnormal glycosaminoglycan catabolism of Hurler or Hunter (mucopolysaccharidosis I and II) syndrome patients fibroblasts could be normalized by the addition of "corrective factors" to the medium (Fratantoni et al., 1968; Fratantoni et al., 1969; Neufeld and Fratantoni, 1970). Although this corrective factors were presumed to be the missing enzyme, Porter et Al. in 1971 gave the first proof of the whole concept showing that arylsulfatase A, a lysosomal enzyme deficient in metachromatic leucodystrophy was capable of degrading stored cerebroside sulfate in patients fibroblasts (Porter et al., 1971). The enzymes L Iduronidase and iduronidate sulfatase were then shown to be the corrective factors respectively for Hurler and Hunter patients fibroblasts (Bach et al., 1973; Bach et al., 1972). Later on, a specific signal required for endocytosis was discovered, the mannose-6-phosphate on the carbohydrate chains of lysosomal enzymes, (Kaplan et al., 1977a; Kaplan et al., 1977b). Not only the cellular endocytosis of lysosomal enzymes is a receptor mediated process, but mannose-6-phosphate signal was demonstarted to be also required for intracellular targeting to lysosomes (Fischer et al., 1980).Tissue culture experiments with various lysosomal enzymes then demonstrated that very little amount of enzyme (less than 5% of normal intracellular activity) could allow correction of deficient cells (Cantz and Kresse, 1974; Porter et al., 1971). All this studies together provided the rationale for early clinical studies of ERT in lysosomal disorders.

We will focus here on research and clinical applications for enzyme Therapy in mucopolysaccharisosis (MPS) with central nervous system (CNS) involment, mainly MPS I and MPS IIIA (although MPS II, MPS VII, and all MPS III dysplay major neurological manifestations). The current status of ERT in MPS disorders with central nervous system involvement is listed below:

MPS I enzyme, L Iduronidase, is currently a standard care for somatic manifestations of the disease. It has been approved for use in USA and Europe in 2003 (Aldurazyme, Biomarin). There is one ongoing clinical trial to explore its clinical use by intrathecal administration.

MPS II enzyme, iduronidate sulfatase, the approval is currently under examination by regulatory agencies in USA and Europe (I2S, TKT-Shire). Ongoing clinical trials are planned for examination of the use by intrathecal administration.

MPS III A enzyme, sulfamidase, is currently under late stage development at TKT-Shire company. More pre-clinical studies are needed for its CNS delivery.

Research work is ongoing on MPS IIIB enzyme, N-acétyl glucosaminidase or NAGLU, (Yu et al., 2000) and MPS VII enzyme, glucuronidase, (LeBowitz et al., 2004; Vogler et al., 2005).

IV.2 ERT for Hurler Syndrome

Also Hurler syndrome is characterised by both somatic and central nervous system manifestations, patients with milder forms of the disease, (Hurler-Sheie and Sheie syndrome) and with residual enzyme activity display little or no nervous neurologic symtoms. Mental retardation is present only in the most severe form of L Iduronidase deficiency, Hurler syndrome. Pre-clinical tests in the canine model of MPS I gave very promising results in terms of enzyme uptake, reduction of storage, and general well being of the animal (Kakkis et al., 1996; Shull et al., 1994). Ten MPS patients, mostly with the intermediate form of the disease, treated in the first clinical trial, showed improvement in biochemical and clinical markers, together with general improvement in their quality of life. Since 2003 and the approval of Aldurazyme, a great number of patients have been treated by intravenous administration, with confirmation of the benefit on somatic symptoms (Wraith et al., 2004). Although a certain immune reaction takes place in the dog model and in the patients, the clinical benefits are still there, and a process of immune tolerance has been observed in long term treated patients (Kakavanos et al., 2003). Despite the efficiency of the recombinant enzyme to reduce storage in many tissues and to alleviate the clinical symptoms of somatic disease, the question of treating the CNS is still a major problem as there is no alleviation in CNS symptoms in the most severe form of the disease, Hurler syndrome. Kakkis and coll. and have chosen to explore the efficiency of intrathecal (IT) injections as a route of administration of recombinant L Iduronidase with the aim of treating the brain (Kakkis et al., 2004b). In the dog model, this administration is carried out in the Magna Cisterna, because it's more convenient to do so in this animal, and it is assumed that this is a model of intrathecal injections at the lombar level of the spinal cord in humans. This mode of injection consists in delivering the enzyme into the cerebro-spinal fluid (CSF). The authors hypothesise that "the application of a large concentration gradient should be sufficient to force small amounts of enzyme pass the the brain surface and drive the diffusion of the enzyme into the brain". The dose response study consisted of low, medium and high doses administered intrathecally once per week for four weeks. Two

dogs were treated at each dose level. Surface and deep brain tissue were assayed separately 48h after the last dose. The IT treated dogs had 5.6, 7.5 and 18.9 fold the enzyme levels of untreated or vehicle- treated animals for the low, medium and high levels respectively, at the surface of the brain, and approximately half of these quantities in the deep brain samples. (The corrective concentration of this enzyme is in the range of 2-5% of the normal). Near maximum deep brain levels were achieved at the medium dose, 1 mg, which was selected for further study.

Four MPS I dogs were then treated with 4 weekly doses of 1 mg administered via intracisternal injection and studied again at 48h. Significant decrease of GAG storage was measured in the total brain and was reported to be similar of the GAG level in control dogs.

Although these results are very encouraging, the problem of having to repeat regurlarly the injections still remains. The second difficulty is the development of a dose dependant immune response by the treated dogs and a marked meningeal inflammation. Further unpublished studies by Kakkis and coll. have show a clear also partial improvment of GAG storage in the whole brain three month after different regimen of medium dose treatment (Le et al., Abstract of the American Society of Human Genetics- ASHG- 2005 meeting). On the other hand, the team continues to work on the immune reaction question and had suceeded in tolerisation of dogs (Kakkis et al., 2004a). Tolerisation protocols need more investigation and are currently difficult to transpose to the clinic. The IT administration is currently under clinical trial for Hurler and Hurler-Sheie patients with major endpoints being spinal cord compression, alleviation of hydrocephalus and the thickness of meninges. Indeed the spinal cord compression results at least in part from meningeal storage, which was totally alleviated in the dog experiment. Another abstract of the 2005 ASHG meeting indicates improvment of spinal cord compression in one bresilian patient (Giugliani et al.). No adverse effect was observed. A second patient is currently treated in UCLA (Patty Dickson, personal communication). To date, this is the only clinical trial aimed at treating neurological manifestations in an MPS disorder.

IV.3 Towards a clinical trial for sulfamidase replacement therapy in the CNS

The human sulfamidase gene has been discovered by John Hopwood's group in 1998 (Scott et al., 1995) and the recombinant enzyme was soon produced, purified and caracterized by the same group (Bielicki et al., 1998).

Again from that group, a single study of intracerebral sulfamidase administration in the mutant adult mouse brain is available to date (Savas et al., 2004). In these experiments, a quantity of 2 microliters of the enzyme (17 microgram/microliter) is administered bilaterally into the Dentate Gyrus of the Hippocampus and the cerebellum, i. e. four different injections sites, in an

attempt to target the whole mouse brain. The experimental design includes different treatment groups at 6, 12 and 18 weeks, with sacrifices at 24 weeks, i.e. 18, 12 and 6 weeks after treatment respectively. (Total mice treated is 9, 3 per groups). The chosen endpoints are an ultrastructural study (electron microscopy) of brain parenchyma in the two injected neuroanatomical regions, hippocampus and cerebellum for an examination of the vacuolation and storage inside neuronal and glial either perivascular or free. Biochemical marquers used for light microscopy are the ubiquitin and glial fibrillary acidic protein (GFAP) antibodies. The ubiquitin staining allows visualizing dot like structures (DSL) indicative of neurodegeneration. On the other side, GFAP staining would give indication of the reactive gliosis which is a signature of neuronal dysfunction/death.

In brief, the treatment is able to reduce storage, gliosis, and to a certain extent the number of DSL around the injection sites, but with no efficiency at all in some specific zones displaying marked storage and neurodegeneration in control mice. This zones near or inside the brainstem seemed to be particularly vulnerable to the disease process.

Treatment outcome depended on the time between treatment and sacrifice. The best results could be obtained on gliosis and diminution of storage and vacuolation on mice treated at 18 wks (6 wks before examination) but neurodegeneration signs (DSL visualised by ubiquitin staining) were not reversible at this stage. Mice treated at 6 weeks showed diminished neurodegeneration but important amount of storage, presumably due to re - accumulation since treatment.

This results show that the enzyme is able to have a partial therapeutic effect when injected directly into the brain parenchyma of mice. It also demonstrates the possibility to prevent some neurodegenerative lesions by providing enough active recombinant enzyme. This procedure is unlikely to be amenable to the clinic in the form of multiple intraparenchymal injections into the brain. The design of the study makes it difficult to extrapolate because this delivery process used, which does not represent an acceptable route of repeated administration in human. although we already know that intraveinously infused recombinant enzyme does not reach the central nervous system (Gliddon and Hopwood, 2004), we still need comprehensive studies with the sulfamidase enzyme to characterize the outcome of intracerebroventricular versus intrathecal (lombar) administration. This two administration routes, if efficient, should be amenable to the clinic, even if the former one would necessarily implicate intracerebroventricular permanent devices in order to allow repeated administration (Medtronic). Specific studies are also needed for the use of such devices. According to this single study, and remembering that the data were obtained by an intraparenchymal administration of the enzyme in the mouse brain, the frequency of administration should be at least once every 6 weeks, but this is a gross evaluation as the study was not designed to directly answer the question of administration frequency, but

rather to assess the possibility of preventing intracerebral lesions and examining their reversibility after intraparenchymal ERT.

There is no more data to give an idea of the frequency needed for the prevention or stabilisation of intracerebral lesions neither in mutant mice or mutant dog brain. Ongoing studies are carried out in John Hopwood's group in Adelaide (personal communication), and will probably provide more insights on the faisability and efficiency of ERT with this particular enzyme. The diffusion, endocytosis and lysosomal targeting of each enzyme should be different and need specific evaluation. For example, in general, very little amount of exogenous lysosomal enzyme are necessary for correction of patients cells in vitro, but there is no direct evidence of the amount needed with the sulfamidase recombinant enzyme. In general, direct intracerebral administration of lysosomal enzyme is poorly studied precisely because of the major roadblock of repeated administration. The only work published to date addressing the question of intaparenchymal diffusion of a lysosomal enzyme from an intracerebroventricular injection site is a study in the normal rat brain (Belichenko et al., 2005). In this study on wild type rats, human □ L Iduronidase spreading was measured in the brain parenchyma from a single intraventricular delivery site. The authors found that immunocytochemistry allowed detection of the enzyme at a maximal distance of 2mm far from the injection site and at a mean distance of 1,2 mm. Even if this results are not really encouraging, similar studies should be carried out in the sulfamidase mutant mice and mutant dog brain with the sulfamidase enzyme with subsequent examination of the outcome on lesions in various neuroanatomical regions. Very little amount of enzyme are unlikely to be detected by immunohistochemistry even if it could have a therapeutic effect. Again with □ L Iduronidase, a recent study demonstrate the anterograde and retrograde transport of the enzyme inside neurites (Chen et al., 2006). This mechanism should allow the enzyme to be available and spread very far from a single source in the brain. This mechanism is not demonstrated for the recombinant sulfamidase to date.

A great effort should be made on dose response studies in the mutant dog brain, together with investigations on administration route with lombar and/or intracerebral devices and frequency regimen.

The challenge of targeting the brain should not mask the unmet need of treatment for peripheral symptoms in Sanfilippo disease, even if somatic symptoms are mild in that MPS in comparison with Hurler syndrome for example. Once the enzyme sulfamidase available, it should be anyway find its first therapeutic usefulness as a treatment for the somatic symtoms by intraveinous administration. On this side, the considerable amount of data accumulated by clinical trials in Hurler and Hunter diseases and the current clinical use of ERT in the former one should provide enough knowledge to help accelerate the approval process once the safety issues have been addressed. Moreover, a recent study in the mouse model of MPS VII indirectly suggested that very high enzyme concentrations in peripheral blood should lead to a partial availability of the

enzyme □ glucuronidase inside the brain through unknown mechanisms (SLY, PNAS). Interestingly, the particular mouse model used in this study does express a mutated human □ glucuronidase gene and is therefore immunologically tolerant to the enzyme. Even if the very high doses (2 to 20 mg/kg) used in this study are not reasonably transposable to humans, the elucidation of the mechanism by which the enzyme has access to the CNS in these mice would be of great interest.

V. THERAPEUTIC POTENTIAL OF ESTROGENS IN SANFILIPPO SYNDROME

V.1 Estrogen and progesterone treatment of Sanfilippo patients

Two case reports have mentioned an improvement of behavioural symptoms in MPS III A patients with an estrogen treatment. Psychomotor and self injurious behaviour were alleviated in a young adult twenty years old Sanfilippo type A female patient (Kushner and Guze, 2005). In a previous report, aggressiveness was also shown to be alleviated in two Sanfilippo type A female patients in an earlier study (Hier et al., 1999). In Kushner and guze's study, the 20 years old patient goes to school. This could be in favour an attenuated form of the disease, although the nature of her genotype is not known. Nevertheless, she showed important improvement of her psychomotor agitation, self mutilation and sleep disturbance behaviours as soon as 72h after the initiation of the treatment. This treatment consisted of a daily ethinyl estradiol (30 microgrammes) and norethinedrone acetate (0,3 microgrammes) regimen, which is qualified as a low dose treatment by the authors. They reported these symptomatic improvments to be maimtained for six weeks, and no more information is given on the long term outcome of the treatment and further evolution of the disease in this patient.

Alghough these observations are of great interest, mainly because they are case reports of patients' studies, a lot of questions still remain unanswered. Moreover there is no study currently available on MPS mice model for the investigation of the therapeutic potential of hormonal treatments in the context of MPS diseases. No more data are available on the possible mechanism of the described effects in human patients. As proposed for the *in vitro* effect of genisteine on patients fibroblasts (see below), one can speculate that an inhibition mechanism of GAGs synthesis could be at the basis of these observations. Estrogens have also been shown to display important neuroprotective potential in various models of neurodegeneration. However, as the effects observed on this patient are seen as early as 72h after the initiation of treatment, they can hardly be attributable to mechanisms opposing the neurodegenerative processes. These hormones have pleiotropic effects on the cell and on the frmale organism physiology, including gene expression regulation and indirect control of signal transduction by growth factors. The latter phenomenon may be implicated in a potential and transitory therapeutic effect of estrogens in Sanfilippo syndrome, as a perturbation of signal transduction processes is probably triggered by abnormal heparan sulfate species resulting from impaired degradation.

V.2 Substrate reduction therapy approach with the isoflavone genisteine

Genisteine (4', 5, 7-trihydroxyisoflavone or 5, 7-dihydroxy-3- (4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) potential as an inhibitor of GAG synthesis has been recently investigated on MPS patients fibroblasts. The effect of this isoflavone compound is also interpreted by Dr Wegrzyn and his group as a "gene-expression targeted isoflavone therapy" (Piotrowska et al., 2006) . To counteract the impaired degradation of the glycosaminoglycans, the aim of this approach is to obtain an inhibition of their synthesis. The theoritical basis of this approach lies on the following claims:

1) Maximum synthesis of GAGs requires Folliculostimulating Hormone (FSH) or Epidermal Growth factor (EGF) to reach a maximal level (Pisano and Greene, 1987; Tirone et al., 1997).

2) The EGF transmembrane receptor is phosphotylated display a protein kinase activity, leading to a cascade of kinases activation and to subsequent gene regulations. This tyrosine-specific protein kinase activity of the EGF receptor is inhibited by genistein (Akiyama et al., 1987).

The authors have treated MPS patients fibroblasts in culture, and performed an evaluation of GAGs synthesis by measurement of (^{35}S) SO_4^{-2} uptake. In mammalian cells, SO_4^{-2} is incorporated almost exclusively into GAGs. Synthesis of GAGs seems to be inhibited in MPS I, MPS II, MPS IIIA and MPS IIIB and wild type fibroblasts. An electron microscopy study was also performed on MPS I fibroblasts and seemed to show a disappearance of intracellular abnormal structures linked commonly observed in the disease.

These very preliminary results indeed need more investigations. *In vivo* experiments on mice model of different MPS forms should confirm or not the interest of such compounds in these different forms of pathological GAG storage. As genistein is able to cross the blood brain barrier, its effect obviously deserve comprehensive studies in MPS IIIA and IIIB mice models. Ongoing experiments are performed in Dr Wraith's group, Manchester, UK). If a therapeutic effect is seen in mice, further dog experiments should make a toxicity study and the dose range study available for the design of a clinical trial. As said below, the pleiotropic effects of oestrogen on the cell physiology and metabolism could lead to a transitory alleviation of GAG storage in the lysosome, although it is hardly imaginable that it could constitute a long term curative therapy for Sanfilippo syndrome.

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