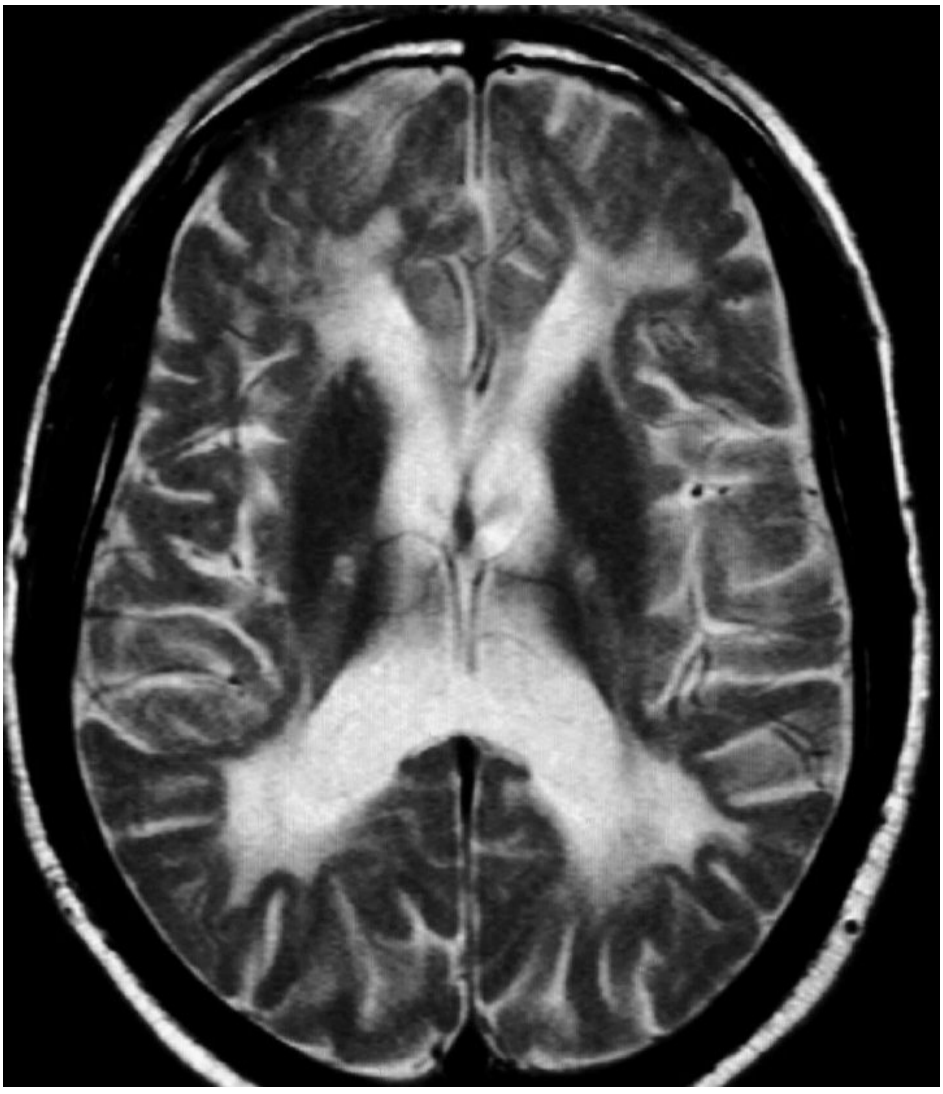
# Developing treatment options for

# Metachromatic Leukodystrophy



# Overview MLD Network Project


## Summary

This proposal encompasses 8 projects all of which cover treatment options for metachromatic leukodystrophy. These options involve enzyme replacement, gene therapy, neural stem cell transplantation and substrate reduction therapy. Enzyme replacement is already in the early clinical stages. It can be expected that any improvement in enzyme delivery to the brain will be of high relevance for optimizing enzyme replacement therapies and are thus of immediate impact for the patients. Therefore a number of projects of this proposal are concerned with various aspects of enzyme delivery to the brain.
Gene therapy and stem cell transplantations are still in the preclinical and experimental stages, respectively. One project examines the feasibility of neural stem cell transplantation therapies for MLD. Another project is involved in the preparation of hematopoetic stem cell based gene therapy trials in humans scheduled for the near future. Small molecule based treatments have so far not been thouroughly investigated in MLD. Therefore one project aims to develop tools which allow large scale screening for molecules suitable for substrate reduction therapy in MLD. Finally, evaluation of any therapy in clinical trials demands a detailed knowledge of clinical history of MLD to define suitable therapeutic endpoints. Therefore one clinical project aims at precise description of clinical history in MLD patients.
The proposal has a duration of two years.


## Clinical history (Project 1)

Detailed clinical data on the natural history of MLD are necessary for any clinical study evaluating therapies in MLD. Project 1 has already designed MRI and motor scores specifically for MLD patients and will use these scores for a more detailed description of clinical history of MLD. Specifically this project will try to internationalize the existing MLD data bank to enhance the data basis for clinical description of the disease.

## Mechanisms of enzyme replacement therapy ( Projects 2, 3, 4, 5)

Preclinical studies performed in a mouse model of metachromatic leukodystrophy have surprisingly shown that sulfatide storage in brain is reduced upon repeated intravenous injections of high doses of recombinant arylsulfatase A. This suggests that some enzyme may be able to cross the blood brain barrier. The mechanisms and pathways behind this phenomenon are unknown. Project 2 will examine the transcytosis of recombinant arylsulfatase A in a blood brain barrier cell culture model. This model is based on primary porcine brain endothelial cells. Recombinant arylsulfatase A will be added to the apical side of the culture system and the delivery to the basal site after various time periods and enzyme modifications will be determined. This cell culture system should allow to determine the rate of transcytosis and the pathways involved. It may yield clues to improving delivery of enzyme across the blood brain barrier. Whereas project 2 is based on an in vitro cell culture system project 3 will add in vivo information to passage of ASA across the BBB. Another possible route to deliver enzyme across the blood brain barrier is the paracellular route. Project 4 will fuse arylsulfatase A to peptides which bind to proteins constituting the tight junctions between the endothelial cells. The possible transfer of such proteins across the blood brain barrier can be tested in cell culture systems available in projects 2 and 4. Another option for CNS delivery of recombinant arylsulfatase A may be the use of nanoparticles. Recombinant enzyme will be coupled to nanoparticles within project 5 and tested for brain delivery in projects 2 and 3. If expedient, results from project 2, 3, 4 and/or 5 can be tested for therapeutic efficacy in mouse models provided by project 6 in follow up projects.

## Developing substrate reduction therapy for MLD (Project 6)

So far research on MLD has largely ignored the development of therapies based on small molecules. One of the options involving small molecules is substrate reduction. Project 6 aims at the development of tools which shall allow screening for small molecules which can be useful to develop substrate reduction therapy for MLD.

**Neural stem cell transplantation and gene therapy (Projects 7, 8)**

Arylsulfatase A overexpressing cells can be used as enzyme producers supplying deficient resident neuronal and glial cells after transplantation into the brain of arylsulfatase A deficient mice or possibly patients in future. Project 7 will examine the feasibility of neural stem cell transplantation for MLD in a mouse model of MLD.
Project 8 is part of the preparation of a hematopoetic stem cell based gene therapy trial in metachromatic leukodystrophy patients using lentiviral vectors. The clincical trial is scheduled for the near future. The project will assess the biological efficacy and safety of this treatment approach.

**Communication**

The project coordinators will meet personally at the annual B4B meetings. In addition, telephone conferences will be organized every 4 months for internal information about the progress of the projects. This is in particular important for projects 2, 3, 4, 5, and 6 in which results from one project may lead to modifications of the work plan for the other projects. Communication will be organized by the coordinator of project 6.

| Project 1 Ingeborg Krägeloh-Mann Tübingen Germany | Requested Bugdet for 2 years |
|---|---|
| Epidemiology and description of the natural course of metachromatic leukodystrophy manifesting during childhood and adolescence | 110.000 € |
| Project 2 Hans Joachim Galla / Münster / Germany<br>Arylsulfatase A transcytosis across the blood brain barrier | 111.000 € |
| Project 3 David Begley / London / UK<br>Arylsulfatase A transport at the blood brain barrier<br>Can it be delivered to the CNS? | 134.382 € |
| Project 4 Ingolf Blasig / Berlin / Germany<br>Modifying arylsulfatase A with peptides to bypass the blood brain barrier | 80.000 € |
| Project 5 Jörg Kreuter and Gelperina / Frankfurt / Germany<br>Development of functionalized nanoparticles for the delivery of arylsulfatase across the blood brain barrier | 115.000 € |
| Project 6 Volkmar Gieselmann / Bonn/ Germany<br>Development of tools to search for inhibitors of cerebroside-sulfotransferase | 115.000 € |
| Project 7 Maurizio Scarpa / Padova / Italy<br>Angelo Luigi Vescovi / Milan / Italy<br>Neural stem cell treatment for MLD | 120.000 € |
| Project 8 Alessandra Biffi / Milano / Italy<br>Hematopoetic stem cell gene therapy for metachromatic leukodystrophy | 80.000 € |
| Total | 865.382 € |

Recombinant human ASA used within this network will be provided by Shire based on an already signed Material Transfer agreement.

**Project title**

Epidemiology and description of the natural course of metachromatic leukodystrophy manifesting during childhood and adolescence

**Principal Investigator**
Prof. Dr. Ingeborg Krägeloh-Mann, University Children's Hospital Tübingen, Department of Child Neurology, University of Tübingen

**Background**
Metachromatic Leukodystrophy (MLD) is a rare, genetically determined disease manifesting mainly during childhood leading to early death or during adolescence then with a more protracted course. Descriptive analysis of the clinical course of the diseases is of current international interest, especially with regard to emerging therapeutic options such as stem cell transplantation (SCT) and enzyme replacement (ER). Birth prevalence of the condition, especially with respect to a specific clinical course are not clearly known. This project deals with the clinical history of MLD and offers ideal possibilities to study population-based epidemiology, which is relevant also for the afore mentioned questions.
A major goal is to offer access to the national database for the disease to be used on an international level.

**State of research and own previous work**
During the last two years, we actively recruited MLD patients. We currently have natural course data on 21 late infantile and 38 juvenile and adult patients with MLD. We have established a scoring system to describe clinical (gross and fine motor function, GMFCS and BFMF) and neuroimaging features and focused so far on the evaluation of patients with late infantile MLD. . An additional scoring system for the juvenile course has to be established for cognitive, language functions and behaviour, relevant for the early course. Ongoing active recruitment of patients will be necessary for epidemiology and description of the natural course. MRI scoring has to be validated and applied accordingly. Clinical course of MLD after SCT will be descriptively analyzed. Counselling of affected patients and their families is ongoing and a permanent information-forum will be established.
An international consortium has been established on the topic of MLD with the aim to harmonize definition and tools for the description of the disease in order to establish a basis for therapy evaluation which is urgently needed, as there are currently no standards as to whether and when these therapeutic interventions are indicated.

**Aims**
Nation-wide recruitment of MLD patients to establish birth prevalence of infantile and juvenile forms
Description of the natural course in infantile and juvenile MLD including neuroimaging. In addition to motor scores a scoring system for cognitive, language functions and behaviour has to be established, the alteration of which determine the early disease course in iuvenile MLD.
Disease course after bone marrow transplantation
Patient hotline for MLD
To establish and monitor an international part of the data base

**Methods and approach**
Birth prevalence and annual incidence are estimated. Confidence intervals will be calculated based on the Poisson distribution.
Time-to-event analyses will be performed with respect to the development of disease assessed by motor (GMFCS, BFMF) and MRI scores.
Further methods and models of longitudinal data analysis will be used as adequate.
Associations between motor scores and MRI will be assessed.
Possible effects of therapeutic interventions (e.g. enzyme replacement study, stem cell transplantation) and patterns of disease will be described.

**Expected Results**
birth prevalence of infantile and juvenile forms of MLD (estimates: we know of 44 patients with MLD diagnosed between 2000 and 2006 and having an infantile or iuvenile course. The total number of livebirths in Germany during these 7 years is 4.99 million. Thus, we can calcuate a birth prevalence rate of at least 0.88 per 100.000 live births for MLD (which is near to the highest reported prevalence in the literature for Europe, e.g. 1.0 per 100.000 livebirths)
Description of the natural course in infantile MLD including neuroimaging is already ongoing, data reveal a very stereotyped pattern of decline; publication is pending. Iuvenile MLD will be described

according to the motor pattern and a scoring system for cognitive, language functions and behaviour will be established
Disease course after bone marrow transplantation.
Patient hotline for MLD
Establishment and monitoring of the international part of the data base

**Budget**

Total: **110.000 € for two years**.

**Justification of Budget**

Staff:
50% of a medical assistant for the help in patient recruitment and data administration
22.000€ per year

25% MD for patient recruitment, patient contact, data collection, evaluation and publication of results
15.000€ per year

25% of an external monitor to ensure data quality 11.000€ per year

Membership fees for TMF platform 7.000 € per year
TMF is a platform supported by the German Government which provides software tools and solutions to all problems associated with the establishment and maintenance of clinical data bases. These tools are designed such that they implement legal, data protection, and quality control requirements applicable for clinical data bases. Membership entitles to access to tools developed by TMF and can greatly facilitate the establishment of national as well as international data banks.

**Timing**

Descriptive analysis of clinical course in children with a late-infantile form of MLD including MRI analysis (6/2009 - 2/2010)
Expected results: time between first symptoms and diagnosis, profile of first symptoms. Motor and neuroimaging scores over time (Kaplan Meier). Interrater reliability of modified motor scores.

Clinical course of juvenile MLD – data collection and analysis continued. (6/2009 - 2/2011)
Expected results: parallel to 1 and development of a cognitive-communicative score

Continuous information-platform for affected families (6/2009 – 5/2011).
Expected results: reference centre nationwide for diagnostic and therapeutic coordination

Clincal course of patients with MLD after SCT (6/2009 – 5/2011).
Expected results: As mentioned in 1 and 2 under therapeutic intervention.

Access to the data base – translation into English etc. – will be established during the first 6 months as well as the monitoring of the incoming data.

**Qualification for the principal investigator for the project**

The applicant has a longstanding research interest in describing the phenomenology including brain morphology using neuroimaging of chronic motor disorders in childhood such as cerebral palsy (CP); this includes also population based epidemiology as well as studies on brain plasticity. She is in the team management of a EU funded European network on CP running since more than 10 years, where harmonization of definition and classification of CP has been achieved (using standardized instruments) as well as the establishment of a common data base which is running now prospectively. This interest has been enlarged since 10 years and includes sphingolipidoses, especially MLD. She is part of the medical board of therapeutic enzyme replacement studies which are ongoing (clinical phase I/II studies). She leads a laboratory for biochemical diagnosis in shingolipiosis.

**Five selected publications of the principal investigator since 2007**
Kehrer C, Kustermann-Kuhn B, Raabe C, Krägeloh-Mann I. (2008) Natural history of metachromatic leucodystrophy (MLD) – clinical course. Eur J Pediatr 167

Kehrer C, Kustermann-Kuhn B, Krägeloh-Mann I 2008 Clinical course of metachromatic leucodystrophy (MLD) in children - an analysis using standardized functional motor scores. Neuropediatrics 39; DOI: 10.1055/s-2008-1079467
Platt MJ, Cans C, Johnson A, Surman G, Topp M, Torrioli MG, Krägeloh-Mann I Trends in cerebral palsy among infants of very low birthweight (<1500g) or born prematurely (<32 weeks) in 16 European centres: a database study Lancet, 369: 43-50 (2007)
Krägeloh-Mann I, Horber V The role of magnetic resonance imaging in elucidating the pathogenesis of cerebral palsy: a systematic review Dev Med Child Neurology, 49: 144-151 (2007)
Cans C, Dolk H, Platt MJ, Colver A, Prasauskiene A, Krägeloh-Mann I Recommendation from the SCPE collaborative group for defining and classifying cerebral palsy Dev Med Child Neurol Suppl 109, 49: 35-38 (2007)

**Demarcation from other currently funded projects of the applicant**

1, Funding source DFG
Title Reorganisation of the brain after early lesions

This project deals with the young brain's capacity for reorganisation after early lesions and uses neuropsychology and functional imaging such as fMRI, MEG. There is no overlap with this application.

2. Funding source EU DG SANCO
Title Harmonisation and dissemination of best practice and clinical Information in children with cerebral palsy

This project deals with standardized evaluation of clinical and neuroimaging findings in children with CP and their dissemination within a European network and beyond

3. Funding source: BMBF (German Ministry of Science) Leukonet III
Title: Epidemiology and description of the natural course of leukodystrophies with known genetic defects manifesting during childhood and adolescence.

This project deals with MLD and globoid cell leukodystrophy (GCL). The goal is to establish birth prevalence data for GCL and MLD and to describe the natural course of both diseases using standardized scores, description should also included neuroimaging. The monitoring of the data base for the clinical projects of Leukonet III is also task of this project. The funding supports 75% MD and 50% technical assistant for patient recruitment and data documentation as well as 25% data monitoring.

Funding for Leukonet III was cut back. This restriction led to insufficient funding for a considerable part of the projects within the network. This means that part of this project could not be followed such as the set up of an evaluation of the cognitive and behavioural development in especially iuvenile patients. These symptoms precede motor symptoms for a variable period of time. Information on this time window is probably critical for the onset of treatment. There is some evidence that SCT may lead to stabilisation on a high functional level when performed before the onset of neurological symptoms in iuvenile patients. A better knowledge on the period before neurological symptoms occur and gross motor function deteriorates would be most helpful for treatment issues.

The establishment of an international access to the national data base is not funded at all.

Thus, additional funding is critical for the achievement of these aims.

**Declaration of applicant:**
This proposal is not funded by other sources than discussed above and has not been submitted elsewhere

**Project title**

Arylsulfatase A transcytosis across the blood brain barrier

**Principal Investigator**

Hans-Joachim Galla, PhD, Professor, Institute for Biochemistry,
University of Münster, Germany, e-mail: gallah@uni-muenster.de

**Background**

Enzyme replacement therapy within the brain demands for an effective passage of the corresponding enzymes across the blood brain barrier (BBB). This barrier is built up by the endothelial cells lining the inner wall of the cerebral microvessels. These endothelial cells are sealed by tight junctions and thus protect the brain against passive influx of exogenous substrates and also the invasion of blood borne cells.

In our project we aim to investigate the transfer process of the enzyme arylsulfatase (ASA), which is involved in a lysomal storage disease. Preliminary experiments showed that the analytical procedure developed in the partner's laboratory to quantify the human ASA to be used by corresponding antibodies is applicable in our porcine system, since the porcine ASA is not recognized by that antibody. Thus we are able to quantify the transfer of human ASA via the porcine BBB in vitro. We also were already able to show, that an uptake as well a transfer of ASA occurs. The uptake by the endothelial cells was inhibited by Mannose-6-Phospate, but not the transfer. This clearly showed that the uptake is dependent on the Mannose-6-Phosphate-receptor, but not the transfer, which has to be elucidated.

**State of research and own previous work**

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In mammals and man the BBB is formed by the capillaries supplying the brain with blood. At the BBB the capillaries formed by endothelial cells have tight junctions between each cell which block any paracellular pathway. Polar or larger molecules, such as peptides require the specific transport routes in the BBB endothelium (Begley DJ, 2007, in: Enhancement in drug delivery. CRC Press). In the case of enzyme replacement therapy necessary in lysosomal storage diseases the enzymes infused intravenously are too large to penetrate the BBB and transporters for them do not appear to be expressed in the barrier. As a consequence they do not reach brain cells to be taken up and incorporated into their lysosomes. Thus in the context of lysosomal storage diseases the function of the BBB has to be more clearly understood so that effective delivery of therapeutic proteins to the central nervous system can be achieved (Begley DJ et.al., 2008,Current Pharmaceutical Design, 14, 1566-1580). The same is true for the treatment of other brain diseases like Alzheimer, Parkinson or epilepsy. In any case, the therapeutic agent has to cross the barrier. Nanoparticles may be helpful vehicles to facilitate better brain uptake e.g by enforced endocytosis, enhanced membrane permeability or even by Trojan horse mechanisms in case of an artificial inflammation.

We have developed porcine cell culture models for the blood brain barrier as well as for the blood-CSF barrier. The major advantage of these models compared to cell lines is that the cells form very tight junctions (high electrical resistance/low permeability) close to the in vivo values. That has been achieved by the transfer into serum free medium and in case of the endothelial model by addition of physiological concentrations of glucocorticoids (Nitz et al., 2003, Brain Research 981, 30-40). These cells have been successfully applied to study the drug transport across both barriers as well as to study the penetration of inflammatory cells and micro organisms ( Lohmann et al., J.Drug Targeting, 10, 263-276 )Pathogen interaction has been studied using "malaria antigens" obtained from red blood cells infected by plasmodium palsifarum (Treeratanapiboon et al. 2005, BBRC,335,810-818)

Our cell cultures develop high electrical resistances/low permeability (2000 $\Omega cm^2/10^{-7}$ cm/s) in chemically defined medium and thus representing closely the in vivo situation. Endothelial cells respond to glucocorticoids in the physiological range by an increased barrier. Since the formation of tight junctions is crucial for a functional transport in vitro system, our research focuses in parallel on the signalling leading to enforced or weakened tight junctions. We found, that extracellular matrix (ECM) formed by glia cells increases the endothelial barrier. Correspondingly integrins being

responsible for this outside-in signalling are to be identified. Since the ECM is an important extracellular barrier for both, large or cationic molecules as well as leucocytes during inflammation an important focus is the plasticity of the ECM. Matrix Metallo Proteinases (MMPs) are enzymes that degrade the ECM molecules, Tissue inhibitors of MMPs (TIMPs) are their counterparts. Both are produced and secreted by endothelial cells and glial cells. We could show that the equilibrium of MMPs and TIMPs balance the barrier. We could also show that MMPs are able to degrade tight junction proteins thus partially opening the barrier (unpublished results). This balance is controlled by glucocorticoids. Comparable observations have been made with the epithelial cells of the choroid plexus, where however the ECM is produced by the epithelial and the underlying (blood side) endothelial cells.

Both models are also used to study inflammatory processes and the penetration of inflammatory cells on a molecular level. Micro organism's penetration is considered as well.

Another major focus are the efflux pumps of the multi drug resistance, where we have recently discovered a new transporter called ABC G2 (also breast cancer resistance protein, BCRC) at the blood brain barrier (Eisenblätter and Galla, 2003, Brain Research, 971,221-231). Our aim is to understand the molecular mechanisms of substrate specificity and transport characteristics of this family of transporters. Especially the involvement of ABC transporters in inflammatory processes is becoming an exciting field.

Different techniques have been applied to investigate the cellular barrier properties including biochemical assays, proteomics, genomics and especially biophysical techniques. Impedance spectroscopy is one technique which sensitively monitors the barrier properties. Confocal Laser scanning microscopy as well as electron microscopy might be used to study the endocytotic passage. In addition we have scanning force microscopy, a high resolution technique which allows us to scan surface topologies.

Our particular interest is focussed on the mechanisms that control the trans-cellular passage at the blood brain and the blood CSF barrier. Important aspects presently investigated are

- Regulation of tight junction permeability
- Cellular interaction in the neurovascular unit (astrocytes ,pericytes)
- Extracellular matrix and barrier properties
- Inflammation and leucocyte penetration
- Interaction and invasion of microorganism
- Transporter of the Multi Drug Resistance

**Experimental approach**

In our project we aim to investigate the transfer process of the enzyme arylsulfatase (ASA), which is involved in a lysomal storage disease. Preliminary experiments showed that the analytical procedure developed in the partner's (Gieselmann) laboratory to quantify the human ASA to be used by corresponding antibodies is applicable in our porcine system, since the porcine ASA is not recognized by that antibody. Thus we are able to quantify the transfer of human ASA via the porcine BBB in vitro. We also were already able to show, that an uptake as well as a transfer of ASA occurs. The uptake by the endothelial cells was inhibited by Mannose-6-Phospate, but not the transfer. This clearly showed that the uptake is dependent on the Mannose-6-Phosphate-receptor, but not the transfer, which has to be elucidated.

The aim of this proposal is:

- To characterize the uptake mechanism of the ASA by the endothelial cells on a molecular basis. Receptor-mediated as well as adsorptive mediated mechanisms will be considered. Clathrin coated vesicles as well as secretory vesicles might be involved.
- To follow the way of ASA through the cells and the fate of the ASA in the cells. The targeting of ASA has to be followed possibly via early endosomes to late or recycling endosomes as well as lysosomes.
- To investigate the transfer mechanism of that fraction of enzymes that is released on the basolateral side of the cell culture.

- To modify the sugar moieties of the enzymes or the enzyme itself by target sequences and to investigate its consequence on the transport properties.

The overall goal is to improve the therapeutic enzymes in order to facilitate a more effective transfer to the brain.

**Timing**

Since the porcine cell culture system is well developed in our laboratory, the experiments will start immediately. The same is true for the astrocytic coculture. We have however to improve the pericyte coculture and may want to test also a tri-culture system. In addition the effect of a preformed extracellular matrix on the transport process has to be investigated. Within the cell we have to follow the transcellular way and to exclude paracellular diffusion. Within the first year we will identify the ways of passage of the unmodified enzymes. In the second year we will optimize the efficacy of the transfer by improving the uptake or the transcellular trafficking depending on the mechanisms that have been evaluated. Moreover modified enzymes will be applied. At the end of the 2 years period we expect to have clear evidence about the mechanism and how to improve the passage of enzymes across the blood brain barrier in order to give suggestion for an improved enzyme replacement strategy.

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**Expected results**

In collaboration with the partners (Gieselmann,Blasig) we expect to unravel the pathways of transport of the enzyme Arylsulfatase (ASA) or its corresponding modified derivatives over the blood brain barrier from the apical side (blood side). Uptake studies will be performed to show the involvement of the Mannose-6-Phosphat (M-6-P)-receptor. Thus the presence and the expression level of this receptor have to be investigated. We already know that part of the applied enzyme enters the cell by another mechanism which is M-6-P-receptor independent. Within the cells we expect according to preliminary results two ways to be taken by the enzyme. One seems to be a direct passage releasing enzyme into the basolateral compartment, which here represents the brain side. Large amounts of enzymes however are not released from the cells and may be taken up by the endothelial lysosomes. We expect that modification of the enzyme with respect to the oligosaccharides or after modification with a toxin residue may enable transcellular transfer and thus will increase the efficacy for an enzyme transfer to the brain. Corresponding in vivo experiments performed by the partner will verify the in vitro study.

**Budget**

Total: 111.000 € for two years.

**Justification of Budget**

**A) Staff: 2 years PhD student** **60.000€**

Basic equipment for the project is available in the laboratory of the applicant, techniques are established. Therefore the project needs only a PhD student for the duration of two years. A Ph. D student costs about 30.000 € per year, thus a total of 60.000 € is required for two years

**B) Consumables per year**

| | |
|---|---|
| Isolation of cells, Chemicals/Plastic for culture of primary cells (e.g.Percoll, centrifuge cubes, culture dishes ) ,1 preparation/weak | 4.000 € |
| Tissue culture media | 3.000 € |
| Plastic ware | 3.000 € |
| Primary and secondary antibodies for cell characterisation | 2.000 € |
| Filters for permeation studies 8-10€ per filter, 500 filters | 4.500 € |
| ECIS Arrays for TEER measurements | 3.000 € |
| Radioactive tracers for permeability control | 1.500 € |
| General chemicals | 3.000 € |
| **Sum consumables** | **24.000 €** |

**C) Travel** **3.000 €**

Needed to visit of revelant meetings (PhD student and applicant) as well as travel to partners for project discussion

Needed to visit of revelant meetings (PhD student and applicant) as well as travel to partners for project discussion

**Qualification for the principal investigator for the project**

The applicant has a longstanding research experience in the use of cell culture systems to investigate the development and maintenance of the blood brain barrier. Endothelial monocultures as well as cocultures with astrocytes and/or pericytes have been established. Those cultures have been successfully used. In an ECVAM project where the applicability of different cell culture systems as models to study transport across the blood brain barrier has been compared (Prieto et al.,Blood-brain barrier in vitro models and their application in toxicology. The report and recommendations of ECVAM Workshop 49., Altern Lab Anim. 32, 37-50). Special knowledge has been gained in the field of ABC transporters at the blood brain barrier, the role of the extracellular matrix as well as MMPs and TIMPs on the barrier tightness and the penetration of leucocytes through the inflamed endothelial barrier.

**Five selected publications of the principal investigator since 2004**

S. Schrot, C. Weidenfeller, T.E. Schäffer and H.-J. Galla (2005), Influence of hydrocortisone on the mechanical properties of the cerebral endothelium in vitro,
Biophys. J., 89, 3904-3910

C. Weidenfeller, S. Schrot, A. Weidenfeller and H.-J. Galla (2005), Murine brain capillary endothelial cells exhibit improved barrier properties under the influence of hydrocortisone
Brain Res 2005, 1053, 162-174

C. Hartmann, A. Zozulya, J. Wegener and H.-J. Galla (2007), The impact of glia-derived extracellular matrices on the barrier function of cerebral endothelial cells: an in vitro study
J. Exp Cell Res., 313, 1318 – 1325

P. Zeni, E. Döpker, U. Schulze-Topphoff, S. Hüwel, T. Tenenbaum and H.-J. Galla (2007)
MMPs contribute to TNF-$\alpha$-induced alteration of the blood-cerebrospinal fluid barrier in vitro
Am J Physiol Cell Physiol 293; 855-864

A. Zozulya, C. Weidenfeller and H.J. Galla (2008)
Pericyte-endothelial cell interaction increases MMP-9 secretion at the blood-brain barrier in vitro
Brain Res., 1189; 1-11.

**Demarcation from other currently funded projects of the applicant**

1. Funding source: DFG (German Research Council)
   Title: SP1313, DFG Priority Programm, Bio-Nano-Response
   This project deals with the interaction of nanoparticles with lung surfactant lipid-peptide-monolayers and does not interfere with the lysosomal storage diseases

2. Funding source: Industrial Partner
   Title: Use of Nanoparticles for efficient transfer across the blood brain barrier in vitro
   Basic research on the transfer of Nanoparticles across the blood brain barrier. Not related to transfer of lysosomal enzymes

3. Funding source: Fond der chemischen Industrie
   Title: Reconstitution of ABC Transporters into supported lipid bilayers and general support of our research
   No overlap with the project proposed here.

**Declaration of applicant:**

This proposal is not funded by other sources and has not been submitted elsewhere

**Project Title**

**Arylsulphatase transport at the blood-brain barrier in vivo.**
**Can it be delivered to the CNS?**

**Principal investigator**
David J Begley, Blood-Brain Barrier Group, Pharmaceutical Sciences, Hodgkin Building, Guys Campus, Kings College London, SE1 1UL, UK..

**Background**
The blood-brain barrier (BBB) is formed by the endothelial cells forming the microvasculature of the brain. The principle function of the BBB is to preserve a very tight homeostatic control of the extracellular compartment of the brain so that the synaptic and integrative function of the neural cells is optimised and preserved. The BBB is also neuroprotective in that it prevents many neurotoxins, either endogenous or acquired from the environment, from entering the brain. The cerebral endothelial cells are joined at their margins by tight junctional complexes which abolish any paracellular pathways between the endothelial cell so that all molecular movement into and out of the CNS must be by a transcytotic route across the endothelial cells. Lipid soluble small molecules can diffuse across the BBB but many of these molecules are effluxed by ABC transporters present in the barrier (Begley 2004). More polar small molecules are either excluded from the CNS or may be substrates for a variety of solute transporters which are expressed at the BBB (Begley 2007). Large molecules such as proteins and peptides are either excluded from the brain by virtue of their size or are specifically transported across the barrier by means of endocytic receptor-mediated transcytosis mechanisms. Thus the BBB is a major impediment to the delivery of many therapeutic drugs designed to treat CNS disease and may lead to the abandonment of their development. It is generally thought that most current enzyme replacement therapy for lysosomal storage diseases is prevented from reaching the brain for the above reasons, especially as the mannose-6-phosphate receptor expressed at the BBB on the neonatal mouse disappears in the first two weeks of life (Urayama et al. 2008). Recent studies by Matzner et al (2009) have shown that in arylsulphatase knock-out mice intravenously treated (2 times per week, 50mg/kg from 6 to 7 weeks of age) with recombinant human arylsulphatase (rhASA), will reduce storage in the CNS and spinal cord by 34 and 45% respectively compared with non-treated controls. This data is strongly suggestive that rhASA is reaching the central nervous system in therapeutically effective quantities. Given that the mannose-6-phosphate receptor endocytic mechanism is down regulated by 2 weeks of age in the mouse (Urayama et al. 2008), presumably another mechanism of transcytosis across the BBB is operating in these mice which may transport ASA into the brain. The purpose of this proposed study is to explore and quantify the possible mechanism of transport of rhASA across the blood-brain barrier of the mouse and to examine ways in which delivery of enzyme replacement therapy to the CNS might be improved.

**Research Plan**
We have in our laboratory very sensitive in situ perfusion techniques and whole animal techniques to measure BBB permeability and transport parameters for a wide variety of solutes including macromolecules and proteins. We have recently obtained data in mouse models of Sanfilippo syndrome (MPS III) showing significant changes in BBB function as the disease progresses. These techniques will enable us to determine the kinetics and mechanism of rhASA transport across the mouse BBB in detail. The Institut für Physiologisch Chemie of the Rheinische Friederich-Wihelms-Universität Bonn, together with Zymenex A/S Denmark will supply rhASA. The rhASA will be tagged with $^{125}$I using the Iodobead method so that its transport across the BBB can be quantified. This labelling method has been shown not to affect the transport properties of other lysosomal enzymes into the CNS (Urayama et al. 2008). A number of receptor-mediated transport processes for large molecules remain at the BBB in postnatal life (Begley 2007) and the interaction of rhASA with these transporters will be examined. If particular known macromolecule transporters are being used by rhASA to enter the CNS competition by the natural substrates of the transport process in question will be demonstrable and this phenomenon can be used to identify the transporter. It will thus be possible to determine which transport processes rhASA may use to enter the brain. WE will study rhASA transport in neonatal mice (2 days) weanlings and young adults 6-7 weeks. An improved knowledge of the transport mechanism for rhASA will then allow better strategies for the delivery of this, and other ERT, to the brain to be devised and for treatment to be optimised. We at present collaborate very closely with Professor Kreuter at the Göthe Universität, Frankfurt, also a collaborator in this research project, and we will examine the nano-

particle delivery systems that his laboratory can offer with our methods for quantifying BBB transport of rhASA. These nanoparticle vectors may prove a very useful strategy for increasing rhASA ERT delivery across the BBB (Zensi 2009). It will be impracticable in the two years of this project to set up a colony of MLD mice in London so once the interaction of rhASA with the BBB of the normal C57B mouse has been established we will arrange to ship some MLD mice directly to London to repeat the key experiments in the MLD mouse. It is also planned for the research assistant to visit Bonn regularly to collect tissue and endothelial cells from the MLD mouse colony for rtPCR studies. We are currently examining mRNA expression in the brain and BBB of our MPS III mice and the mRNA expression for a number of structural proteins and transporters in the BBB are changing during the course of the disease. It will be very informative to compare this MPS III data with mRNA measurements in the MLD mice. We also have a collaboration with the pharmaceutical company Angiochem and we will seek permission from them to link rhASA to their peptide vector Angiopep-2 to see if this targetor facilitates transport of rhASA across the BBB (Demuele 2008).

**Budget over 2 years**

| | |
|---|---|
| Post doctoral research assistant salary (50% over 2 yr) | 45,000€ |
| Mice 360 C57B @ 16.50€ | 5,940€ |
| Maintenance of C57B mice @ 0.47€/day average 10 days | 1,692€ |
| Transportation of MLD mice from Bonn to London | 2,750€ |
| Consumables and laboratory chemicals | 50,000€ |
| rtPCR materials and genomic studies | 25,000€ |
| Computing and stationary | 2,000€ |
| Travel and attendance at meetings | 2,000€ |
| **Total** | **134,382€** |

A post doctoral worker will be needed for this project as we cannot contract a PhD student for less than the statutory three years of training. A suitable postdoctoral research assistant with experience of the in vivo techniques will also get the project up and running faster.

**Qualifications of the principal investigator for the project**

The blood-brain barrier (BBB) group at Kings College London (KCL) is a constituent part of the Pharmaceutical Sciences Research Division. The principal investigator has a long history of studying the BBB extending over 25 years. It is one of the leading groups in this area and is recognized worldwide. The laboratory has a wide experience, using both in vivo and in vitro techniques, of BBB transport studies. Current work ranges from a purely scientific study of the function of the normal BBB, to pathology of the BBB in disease. Extensive expertise is available in understanding the problems of drug delivery to the brain to treat CNS disease, and we are frequently consulted by pharmaceutical companies and work in close collaboration with a number of large companies. In recent years we have been studying the involvement of the BBB in mouse models of mucopolysaccharidosis (MPS IIIA and MPS IIIB) and the problems of delivering therapy to the brain. We have in place the necessary Home Office Licences regulating the animal experiments and we also have designated radiotracer laboratories. Together with our BBB experience this enables us to contribute uniquely to this project.

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**Five other selected references of the Principal investigator.**

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Begley DJ. (2007) The significance of the blood-brain barrier for Gaucher Disease and other lysosomal storage disorders. In: Eds. Futerman A and Zimran A. Gaucher Disease, CRC Press, Boca Raton. pp 397-421.

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**Other currently funded work in our laboratory**

1. The Society for Mucopolysaccharide Diseases (UK and Spain)
   "Involvement of the blood-brain barrier in MPS IIIA and IIIB (Sanfilippo Syndrome)"
2. Actelion/ The UK Gaucher Association
   "Blood-brain barrier transport of Zavesca"
3. Ara Parseghian Foundation/Hadley Hope Fund/The Addi and Cassi Fund
   "Study into Niemann-Pick Disease"

**Declaration**

The applicant has no other sources of funding for this application or conflicts of interest.

**Project title**

## Modifying arylsulphatase A with peptides to bypass the blood-brain barrier

**Principal Investigator**
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**Background**
The lysosomal storage disease Metachromatic Leukodystrophy (MLD) is caused by the deficiency of arylsulfatase A (ASA). Substitution of the enzyme is proposed by enzyme replacement therapy (ERT). ERT is limited because of restricted protein delivery to the brain, due to the presence of the blood-brain barrier (BBB). The aim of the project therefore is to overcome the BBB by specific targeting of the enzyme to barrier proteins. In addition, the pathway of the modified ASA through the BBB will be identified.

**State of research and own previous work**
In lysosomal storage diseases, a genetic defect affecting a particular enzyme prevents the clearance of molecules meant for lysosomal destruction. Consequently, respective substrates accumulate within the lysosome leading to a multitiude of clinical symptoms among which neurodegeneration may be the most prevalent. MLD is an autosomal recessive disorder due to ASA deficiency resulting in the accumulation of galactosylceramide-3-O-sulfate (sulfatide) in the central and peripheral nervous system. Demyelination is the main pathological finding, but substantial storage of sulfatides in neurons also occurs, and may contribute to the clinical phenotype including neuronal and glial degeneration.

For an extending list of lysosomal storage diseases, different treatment options are available. Recombinant enzymes are administered intravenously in patients. Bone marrow or haematopoietic stem cell transplantation is effective to some extent. In animal models, the CNS may benefit through gene therapy or repeated intrathecal enzyme injection. Less specific approaches include substrate reduction and chemical chaperons. By ERT, the peripheral disease can be treated but the neurodegeneration continues. The enzymes are often large glycoproteins and unable to cross the BBB in sufficient quantities to reverse the neurodegeneration.

In case of MLD, ERT is in early clinical phases, gene therapy in late preclinical stages and cell transplantation is purely experimental. Little attention has been paid to strategies involving small molecules or molecular chaperones. Repeated intravenous injection of human ASA (hASA) may reduce sulfatide storage and improve the nervous sytem and function. However, the BBB limits the access of the enzyme to the CNS which is primarily affected. The development of efficient approaches for brain delivery of therapeutic enzyme is therefore crucial for a future clinical management of MLD.

The BBB is impermeable for solutes such as respective metabolites or drugs and for substrates of the efflux transporters (multi-drug resistance proteins), despite of those compounds taken up by specific transport mechanisms. The paracellular clefts of the endothelium forming the BBB are specifically sealed by intercellular interactions between claudins forming tight junctions. Claudins are highly dynamic transmembrane proteins with high mobility and short half-life [*FASEB J.* 2008, 22, 146] and play a key role in the regulation of the BBB [*FASEB J.* 2007, 21 3666]. Brain endothelial cells are characterized by a unique combination of claudins 3, 5 and, probably, 1 and 12.

We are able to generate recombinant enzymes as fusion proteins or tagged with peptides in large amounts. The linked protein/peptide can be split off and the enzyme purified easily [*J. Biol. Chem.* 2005, 280, 3747; *Cell. Mol. Life Sci.* 2006, 63, 505; *Ann. N. Y. Acad. Sci.* 2009, in press]. The morphological characterisation of the proteins and peptides in cell barrier systems is carried out by means of fluorescent tags or immunostaining. To investigate targeting of peptide or protein to specific claudins coimmunoprecipitation, peptide library assays or surface plasmon resonance spectroscopy is applied. Recently, we developed peptides binding to the second extracellular loop of claudin-3 in submicromolar concentrations and, to a less degree, to claudin-5. These peptides open the tight junctions resulting in increased paracellular permeability and are endocytosed via claudins [Winkler et al., in revision].

Based on our expertise to generate recombinant fusion proteins (enzymes, tight junction proteins), to clarify the function and regulation of tight junction proteins in the BBB as well as to modify the barrier properties with modulatory peptides our proposal is aimed at:

1. Generation and purification of a claudin modulator peptide (CMP), of recombinant human ASA, and of the fusion protein hASA-CMP.
2. Increase of the delivery of hASA through a cell culture model of the BBB.
3. Clarification of the fate of claudins concerning their ligand CMP with respect to the lysosomal, transcellular, and/or paracellular pathway of hASA.
4. With the gained knowledge, preconditions will be established to improve the enzyme delivery to the brain in Metachromatic Leukodystrophy.

**Experimental approach**

According to the aims metioned obove, we plan four work packages:

1. Generation of recombinant hASA, CMP, and hASA linked to CMP

   The generation of hASA-CMP has been taken up. For the experiments, we are using a vector containing hASA obtained from V. Gieselmann/Bonn (project 4). We are cloning $CMP_{194}$ [Winkler et al., in revision] fused to the C-terminus of hASA. The amino acid sequence will be verified by sequencing of the constructs. Then, the construct will be transfected into Chinese hamster ovary (CHO) cells for posttranslational modification, i.e. glycosylation and formylglycine formation. hASA-CMP will be purified by using one-step affinity chromatography using monoclonal anti-hASA antibodies (provided by project 4). The specific activity of the resulting enzyme preparation will be proven by an combination of an ELISA and activity determination in collaboration with the lab of project 4. If the activity of hASA-CMP should be impaired in comparison to hASA, alternatively, CMP can be conjugated to hASA via a cysteine residue (outside active centre/disulfide bonds) or hASA will be administered simultaneously with CMP (hASA + CMP). CMP will regularly be generated in *E. coli* N-terminally fused via a thrombin cleavage site to glutathione-S-transferase (GST-CMP). After purifying GST-CMP from the cell lysate using glutathione-agarose columns and separation of CMP from GST by incubation with 100 U/ml thrombin, CMP will be obtained in high purity. GST will be washed and, then, eluted by 10 mM reduced glutathione [Winkler et al., in revision]. Recombinant hASA will also be received from project partner 4. The targeting of GST-CMP and hASA-CMP, respectively, to claudins will be verified in tight junction-free and claudin-free human embryonic kidney (HEK-293) cells transfected with claudin-3 or claudin-5 [FASEB J. 2008, 22, 146]. For this purpose, the binding to the claudins will be immunocytochemically detected by confocal colocalization studies compared to non-transfected control cells. To check the binding of CMP containing preparations to the second extracellular loop of the claudins respective mutants in the loop, which do not associate CMP, will also be investigated transfected in HEK cells. As an independent binding assay coimmunoprecipitation will be used [Cell. Mol. Life Sci. 2006, 63, 505]. Alternatively, to $CMP_{194}$, e.g., for modification of the binding strength to claudins $CMP_{116}$ can be considered. The above-named techniques run routinely in the collaborating laboratories.

2. Permeability studies of hASA in a cell culture model of the BBB

   hASA, CMP, hASA-CMP and hASA + CMP will be analysed with respect of its effect on the tightness of a cell culture model of the BBB. This model uses human brain capillary endothelial cells termed hCMEC/D3. The cells will be seeded on a filter support and grown to confluence. After incubation with hASA, CMP, hASA-CMP or hASA+CMP, the transcellular electrical reistance (TER) will be detected [*FASEB J.* 2007, 21 3666]. From preliminary experiments, we expect that CMP-containing incubations result in a transient opening of the cellular barrier as indicated by reduction of the TER. The experiments will be started by testing 1 µM protein concentrations. For active approaches, concentration- and time-dependence will be determined to standardise the administration regime. In addition, the permeation of hASA in the different preparations will be detected by the ELISA in collaboration with project 4. For estimation of the permeation, samples will be taken from the abluminal reservoir after luminal administration of hASA.

3. Identification of the pathway(s) of hASA/CMP through the BBB

   To analyse the transcellular pathway hASA-CMP and GST-CMP, respectively, will be incubated with claudin-expressing and CMP-binding cell cultures (e.g. hCMEC/D3). First, the internalization of the CMP-containing preparations will be monitored by assessing of the intracellular immunostaining using anti-GST and anti-hASA antibodies (X-Y image in combination with the Z-stacks). Thus, it will be proven whether hASA can enter the cells. As abluminal membrane marker Na,K-ATPase is costained to judge whether the enzyme may cross the cells. To characterize the transcellular pathway colocalization studies will also be performed with markers of different cell organells (e.g., lysosomal-associated marker protein 1, cytosolic glyceraldehyde-3-phosphate dehydrogenase). hASA and GST serve as controls.

   To analyse the paracellular pathway the effect of hASA-CMP and CMP will be investigated with respect of the interaction between claudins in cell systems. CMP will be taken as positive, hASA

(GST) as negative control. The trans-interaction of claudins between two opposing cells, primarily tightening the paracellular space, will be shown as enrichment of the claudin in the cell-cell contact between two claudin-expressing cells. The cis-interaction within one cell membran, also influencing the tight junction architecture, will be measured by a fluorescence resonance energy transfer (FRET) assay. This assay uses cells transfected with C-terminally fused claudin-3-yellow fluorescence protein (YFP) and claudin-3-cyano fluorescence protein (CFP), and claudin-5-YFP/CFP [FASEB J. 2008, 22, 146]. Reduction of the contact enrichment and/or FRET efficiency will indicate that hASA-CMP opens the tight junctions to facilitate the paracellular permeation.

4. Suitable hASA preparations will be made available to project partner 4 for validation in a mouse model of MLD [*Hum. Mol. Genet*. 2005, 14, 1139] to verify the delivery to the brain and its antineurodegenerative effect. In addition, hASA will be transferred to project partner 6, J. Kreuter/Frankfurt, M., for loading in nanoparticles as an alternative pathway to circumvent the BBB.

**Timing**

1. Establishing, as far as still necessary, the procedures to generate the recombinant proteins and peptides to produce sufficient amounts and purities will take about one year (collaboration project partner 4). This includes the major characterisation of the preparations (enzyme activity, claudin binding). Where required protein generation will be performed in year two also.
2. Permeability measurements will be executed the second and third half-year after start of the project.
3. Characterisation of the proposed pathways through the cellular layers will be planned for the second year.
4. Suitable hASA approach(es) will be provided for the in vivo testing (project partner 4) at the end of the project. Pure hASA without CMP will be prepared for nanoparticle encapsulation (project partner 6) in year one.

**Expected Results**

1. Production of recombinant hASA, hASA-CMP, CMP, GST-CMP, and GST in high quantity and quality,
   Demonstration that CMP-containing preparations bind specifically to the second extracellular loops of the claudins 3 and 5.
2. Demonstration of improved paracellular permeation of hASA-CMP and/or hASA + CMP compared to hASA,
   Definition of an optimum approach of hASA-CMP and/or hASA + CMP to pass throug a cell cultur model of the BBB
3. Identification of the pathway(s) of hASA-CMP and/or hASA + CMP through the BBB
4. Supply of hASA, hASA-CMP and/or hASA + CMP for nanoparticle preparation and for in vivo validation, respectively.

**Budget**

Total: 80.000 € for two years.

**Justification of Budget**

A) Staff

The techniques to perform the project are well established in the applying laboratory. We therefore do not need an experienced post doc but a PhD student. To execute the work at least two years are required. The costs for a PhD student are 30.000 € per year; thus, the total for two years is **60.000 €**.

B) Consumables for two years

| | |
|---|---|
| Tissue culture media (incl. serum, antibiotics, additives) | 2.000 € |
| Plastic ware (pipettes, cell culture flascs, reaction vessels etc.) | 4.000 € |
| Primary and secondary antibodies (anti-arylsulfatase, labelled sec. antibodies) | 4.000 € |
| Transfection reagents (e.g. lipofectin) | 4.000 € |
| Molecular biological reagents (restriction enzmes, agents for sequencing) | 2.000 € |
| General biochemicals (e.g., buffer components, solvents) | 2.000 € |
| The sum for consumables is | **18.000 €**. |

C) Animal housing
not required

D) Travel
To take part at meetings of the project consortium and to present the results at relevant symposia we apply for **2.000 €**.

**Qualification of the principal investigator for the project**
The applicant has longtime experience in the generation, purification and characterization of recombinant fusion proteins (enzymes, tight junction proteins). This includes the investigation on molecular, peptide, and protein level in vitro, using divers molecular biological, biochemical, and biophysical techniques. In addition, he is well experienced in the generation and establishment of cell culture systems of the BBB. These models, he used to study pathological aspects, such as oxidative stress, to test protective agents. The aim of his research is to develop new pharmacological approaches to modulate the paracellular permeability of the BBB specifically. Thus, and together with the other project partners, specifically V. Gieselmann, all preconditions are given to develop a powerful system to improve the delievry of hASA through the BBB.

**Five selected publications of the principal investigator since 2004**

1. Krause, G., L. Winkler, S.L. Mueller, R.F. Haseloff, J. Piontek, I.E. Blasig. Structure and function of claudins. Biochim. Biophys. Acta – Biomembranes *1778* (2008) 631-645
2. Piontek, J., L. Winkler, H. Wolburg, S.L. Mueller, N. Zuleger, C. Piehl, B. Wiesner, G. Krause, I.E. Blasig. Formation of tight junction: Determinants of homophilic interaction between classic claudins. FASEB J. *22* (2008) 146-158
3. Schreibelt, G., G. Kooij, A. Reijerkerk, R. van Doorn, S.I. Gringhuis, S.M.A. van der Pol, B.B. Weksler, I.A. Romero, P.-O. Couraud, J. Piontek, I.E. Blasig, C.D. Dijkstra, E. Ronken, H.E. de Vries. Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase and PKB signaling. FASEB J. *21* (2007) 3666-3676
4. Blasig, I.E., L. Winkler, B. Lassowski, S.L. Mueller, N. Zuleger, E. Krause, G. Krause, K.H.P. Gast, M. Kolbe, J. Piontek. On the self-association potential of transmembrane tight junction proteins. Cell. Mol. Life Sci. *63* (2006) 505-514
5. Mueller, S.L., M. Portwich, A. Schmidt, D.I. Utepbergenov, O. Huber, I.E. Blasig, G. Krause. The tight junction protein occludin and the adherens junction protein α-catenin share a common interaction mechanism with ZO-1. J. Biol. Chem. *280* (2005) 3747-3756

**Demarcation from other currently funded projects of the applicant**

1. Funding source: DFG (German Research Council)
   Title: Interactions of BBB proteins and their regulation
   Brief description: The proposal investigates the molecular interaction mechanism between the specific tight junction protein occludin and the recruiting protein ZO-1.

2. Funding source: DFG
   Title: Dimerisation concept of transmembanous BBB proteins
   Brief description: This project is proposed to demonstrate and to characterise the dimerisation potential of transmembrane proteins expressed in the BBB.

3. Funding source: DFG
   Title: Modulation of the claudin oligomerization to influence the BBB
   Brief description: Screening of small molecules affecting claudin-5 to select modulators of the BBB with the potential to open/close the BBB for pharmacological purposes.

There is no ongoing research project related to the present proposal. Studies currently performed by project partner 2 are related to the molecular structure of the tightening mechanism of the BBB. In addition, the physiology, pathology and pharmacological modulation of the BBB is investigated. This represents challenges to the ERT of lysosomal storage diseases, including LSD.

**Declaration of applicant**
This proposal is not funded by other sources and has not been submitted elsewhere.

**Project title**

Development of functionalized nanoparticles for the delivery of arylsulfatase A across the blood-brain barrier

**Principal Investigator**
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**Cooperation Partner**
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**Background**

One of the problems in enzyme replacement therapy is that the therapeutic enzymes cannot access the brain because the blood-brain barrier (BBB) prevents their transport. A number of years ago, however, it was shown by Kreuter et al. [*Brain Res. 1995, 674, 171*] that nanoparticles overcoated with polysorbate 80 (Tween® 80) enabled the transport of a hexapeptide, the endorphin dalargin, across the BBB thus yielding significant antinociceptive (analgesic) effects after their intravenous injection. Nanoparticles for pharmaceutical purposes and for medical application are defined by the Encyclopedia of Pharmaceutical Technology [*Encyclopedia of Pharmaceutical Technology, Vol. 10. M. Dekker, New York, 1994, pp. 165*] and the Encyclopedia of Nanotechnology [*Encyclopedia of Nanoscience and Nanotechnology, Vol. 7. American Scientific Publishers, Stevenson Ranch, U.S.A., 2004, pp. 161*] as solid colloidal particles ranging in size from 1 to 1000 nm (1 µm). These nanoparticles after i.v. injection also delivered other drugs across the BBB, including loperamide, doxorubicin, nerve growth factor (NGF), and others, thus enabling significant pharmacological effects of these agents in the CNS. Nanoparticles loaded with doxorubicin led to a long term survival (> 6 months) of up to 40 % of rats in the extremely aggressive intracranial glioblastoma 101/8 model after intravenous injection, whereas the controls died between 10 – 20 days [*Tumori 94 200, 94, 271*]. In order to achieve this significant drug transport across the BBB, coating of the nanoparticles with polysorbate 80 (Tween® 80) or poloxamer 188 (Pluronic® F 68) or the attachment of targeting ligands such as apolipoprotein A-1 or E, transferrin or certain antibodies is necessary. Recent results indicate that the mechanism for the nanoparticle-mediated transport of the drugs across the BBB appears to be endocytosis by the endothelial cells lining the brain blood capillaries and possibly also transcytosis of the nanoparticles [*unpublished results*].

So-far the strategy to employ nanoparticles for the transport of replacement enzymes for the treatment of lysosomal storage diseases across the BBB has not been investigated. However, preliminary information indicates that lysosomal storage disease replacement enzymes also may be delivered across the BBB by nanoparticles. Therefore, in the present project the binding of arylsulfatase A for the treatment of metachromatic leukodystrophy to different types of nanoparticles will be investigated.

**State of research and own previous work**

Biocompatible nanoparticles made primarily by the teams of Kreuter and Gelperina but also by other teams enabled the transport not only of a number of smaller molecular weight drugs across the BBB but also of larger molecules drugs such as dextran 70'000, NGF and the ß-galactosidase reporter DNA, which all retained their activity in the brain after intravenous injection. In addition to these molecules, nucleic acids, genes, antibodies and lipoproteins could be bound to different types of nanoparticles [*Encyclopedia of Nanoscience and Nanotechnology, Vol. 7. American Scientific Publishers, Stevenson Ranch, U.S.A., 2004, pp. 161; Tumori 94 200, 94, 271*]. This binding also protected the latter substances against degradation by enzymes and enhanced their efficacy.

The nanoparticle materials that can be used for brain delivery have to be biocompatible and rapidly biodegradable in order to avoid a prolonged retention of the polymer in the brain. Polymers that meet these requirements include poly(butyl cyanoacrylate) (PBCA), human serum albumin (HSA), and poly(lactic-co-glycolic acid) (PLGA). Since no experience exists with the binding of arylsulfatase A or other replacement enzymes to the nanoparticles it is paramount for the success of the present project to investigate all three nanoparticle types because these polymers have different properties and advantages: albumin nanoparticles are the most versatile particles for the attachment of targeting ligands such as apolipoproteins A-1 or E, transferrin or antibodies, poly(butyl cyanoacrylate) nanoparticles are the most developed for brain delivery, and PLGA is the most promising material with

a lot of material flexibility which is necessary for a successful evaluation of the strategy of employing these carriers for replacement enzymes such as arylsulfatase A. The surface of the nanoparticles then has to be modified by coating with different substances which enabled a transport across the BBB or by the attachment of the above mentioned targeting ligands.

The group of Jörg Kreuter has a longstanding experience (the longest in the world) with the PBCA and the HSA nanoparticles concerning drug transport across the BBB, whereas Svetlana Gelperina has a similar experience with the PLGA and PBCA nanoparticles. Both groups have been working closely together during the last 14 years. As mentioned above, preliminary information indicates that lysosomal storage disease replacement enzymes also may be delivered across the BBB by nanoparticles. For this reason, it is a very promising approach to employ these particles as carriers for arylsulfatase A in the scope of this project.

**Experimental approach**

**The group of Jörg Kreuter** (Frankfurt) will bind the arylsulfatase A to PBCA and albumin nanoparticles overcoated with polysorbate 80 or poloxamer 188, which are the prime coating agents for the BBB transport. The nanoparticles will be characterised concerning drug loading and release as well as concerning their chemical and physicochemical properties. In addition, biological BBB transport ligands such as apolipoprotein A-1 and E as well as transferrin and antibodies will be attached to the nanoparticle surface. It is paramount to the success of the project that different core polymers and surface coats are employed, since drugs bind differently to different polymers, and, in addition, the targeting success not only depends on the polymer used and on the surface properties of the nanoparticles, but also the loaded drugs influence the body distribution.

**The group of Svetlana Gelperina** (Moscow) will focus on the binding of the arylsulfatase A to nanoparticles made of PLGA, a biodegradable and biocompatible polymer that has been approved by the FDA. The use of PLGA allows to vary the rate of particle biodegradation and hydrophobicity, which can be controlled by selecting a polymer of an appropriate molecular mass and desired lactide : glycolide ratio, where lactic acid is more hydrophobic than glycolic acid (the higher the glycolide ratio the faster the degradation). Both parameters are important for controlling the in-vivo behaviour of the particles as well as their loading capacity. Hydrophobicity also is an important parameter to control the time scale of rehydration of cavities when particles are re-suspended in water. The nanoparticles will be prepared using various emulsification techniques (high shear, ultrasonic or high pressure emulsification) or nanoprecipitation. The nanoparticles also will be characterised concerning drug loading and release as well as concerning their chemical and physicochemical properties.

This workplan offers the advantage that it unites the biomedical engineering expertise of the Kreuter's and Gelperina's groups to develop nanoparticles for the transport of replacement enzymes for the treatment of lysosomal storage diseases across the BBB.

**Timing**

In the first half year the binding of the arylsulfatase A to PBCA nanoparticles will be investigated and optimised. During the second half year binding to albumin nanoparticles will be tested. During the second year the targeting ligands apolipoprotein A-1 and E, transferrin, and antibodies will be attached to the surface of these nanoparticles. In parallel arylsulfatase A will be attached to the nanoparticles made of the FDA-approved polymer PLGA. Since this material is much more versatile concerning surface properties and offers a lot of manufacturing and binding opportunities, these investigations require both years. The particles with the best binding and release properties will then be tested in vitro and in animals in the laboratories of Hans Joachim Galla, David Begley, and Volkmar Gieselmann.

**Expected Results**

Optimised nanoparticles with bound arylsulfatase A to PBCA, albumin, and/or PLGA nanoparticles will be developed and characterised, and the binding of this enzyme to the nanoparticles will be optimised. The nanoparticles will then be surface coated or if this does not lead to the required transport properties targeting ligands apolipoprotein A-1 and E, transferrin, and antibodies will be covalently attached to the surface.

**Budget**

Total: 115.000 € for two years.

**Justification of Budget**

A) Staff
All techniques which are needed to perform this project are well established in the laboratory of the applicants. Therefore, the project does not need an experienced post doc but can be performed by 2 Ph.D students as part of their doctoral work. The work does at least need two years. For this costs about 40.000 € per year is required , thus a total of 80.000 € in two years

B) Consumables per year

| | |
|---|---|
| Chemicals | 9.000 € |
| Plastic ware | 3.000 € |
| Antibodies | 2.000 € |
| Albumin | 4.000 € |
| PLGA | 8.000 € |
| Total consumables | 26.000 € |

**Qualification for the principal investigator for the project**

The applicants are working in the area of nanoparticles for 37 (JK) years and 15 years (SG) in the field of nanoparticles for brain drug delivery for 18 years (JK) and 14 years (SG).

**Five selected publications of the principal investigator since 2004**

1. S, C. J. Steiniger, J. Kreuter, A. S. Khalansky, I. N., Skidan, A.I. Bobruskin, Z. S. Smirnova, S. E. Severin, R. Uhl, M. Kock, K. D. Geiger, S. E. Gelperina: Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles. *Int. J. Cancer* 109, 759–767 (2004).

2. B. Petri, A. Bootz, A. Khalansky, T. Hekmatara, R. Müller, R. Uhl, J. Kreuter, S. Gelperina: Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: Revisiting the role of surfactants. *J. Controlled Rel.* 117, 51-58 (2007).

3. J. Kreuter, T. Hekmatara, S. Dreis, T. Vogel, S. Gelperina, K. Langer: Covalent attachment of apolipoprotein AI and apolipoprotein B-100 to albumin nanoparticles enables drug transport into the brain. *J. Controlled Rel.* 118, 54–58 (2007).

4. E. Pereverzeva, I. Treschalin, D. Bodyagin, O. Maksimenko, K. Langer, S. Dreis, B. Asmussen, J. Kreuter, S. Gelperina: Influence of the Formulation on the tolerance profile of nanoparticle-bound doxorubicin in healthy rats: Focus on cardio- and testicular toxicity. Int. J. Pharm. 337, 346–356 (2007).

5. E. Pereverzeva, I. Treschalin, D. Bodyagin, O. Maksimenko, J. Kreuter, S. Gelperina: Intravenous tolerance of a nanoparticle-based formulation of doxorubicin in healthy rats. *Toxicol. Letters* 178, 9–19 (2008).

**Demarcation from other currently funded projects of the applicant**

1. Funding source: Bundeswehr
   Title: Entwicklung von Nanopartikeln als Träger zum Transport von Oximen als Antidote gegen Organophosphatvergiftungen über die Blut-Hirn-Schranke.

**Declaration of applicant:**
This proposal is not funded by other sources and has not been submitted elsewhere

**Project title**

Development of tools to search for inhibitors of cerebroside sulfotransferase

**Principal Investigator**
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**Background**

Approaches to treat Metachromatic leukodystrophy (MLD) encompass enzyme replacement, gene therapy and cell transplantation. Enzyme replacement is already in the early clinical stages, gene therapy is in late preclinical stages and cell transplantation is still purely experimental. For MLD little attention has so far been paid to strategies involving small molecules such as substrate reduction therapy or molecular chaperones. Aim of this project is the development of tools allowing high throughput screening for small molecules to be used in substrate reduction therapy.

**State of research and own previous work**

Enzyme replacement therapy has been or is in the process of being developed for various lysosomal storage diseases. Since most lysosomal disorders are characterized by severe nervous system involvement and the blood brain barrier hinders efficient delivery of enzyme to the brain, alternative strategies must be developed to improve the chances to target the central nervous system. An option is the use of small molecules which are more likely to pass the blood brain barrier or offer possibilities for chemical modification to enhance nervous system delivery, respectively. An approach involving small molecules is substrate reduction therapy. Here the enzymes involved in the synthesis of the compound accumulating in the respective disease is inhibited with the aim to diminish the compound load for the patient [*Acta Paediatr, 2008, 97, 88*]. The biochemical situation in MLD appears very favourable for this approach. Sulfatide is synthesized in a two-step process. First, from UDP-galactose a galactose moiety is transferred to ceramide by UDP-galactose:ceramide galactosyltransferase (CGT). This step takes place in the endoplasmic reticulum. The resulting galactosylceramide is then sulfated by 3'phosphoadenosine 5'-phosphosulfate:cerebroside transferase (CST) acting in the Golgi apparatus. If inhibitors for CST were available, the synthesis of sulfatide could be reduced and the sulfatide load for MLD patients be diminished. There are two prerequisites to find inhibitors for CST: 1. it must be possible to express CST recombinantly and to purify it to an extent which allows for high throughput inhibitor screening and 2. a CST activity assay must be developed suitable for high throughput screening.
CST is a type II membrane bound enzyme with its C terminus facing the lumen of the Golgi apparatus. In order to purify larger amounts of this enzyme, we have deleted the transmembrane region and added various tags to the C-terminus leaving a soluble CST (SCST-tag) which we hoped would be secreted and could be purified from the media of the stably transfected cells via tag-based affinity chromatography. We have pursued this approach since about 18 months but faced an unexpected difficulty. Whenever the enzyme is overexpressed most of the enzyme is retained in the endoplasmic reticulum. To overcome this problem we have fused the CST to various secretory proteins or with transmembrane and cytosolic domains of plasma membrane located proteins to promote exit from the ER. None of our many trials was successful. The data clearly indicate that the structures responsible for ER retention are located in the luminal domain of CST. ER retention limits severely the amount of enzyme we can produce and presents an obstacle for the development of techniques for inhibitor screening. In addition, attempts to express the protein in bacteria and yeast also failed so far. Amounts, however, sufficed to show that soluble tagged CST is enzymatically fully active.
These findings are in accordance with the results from transgenic mice we have generated recently. We produced a transgenic mouse which overexpresses CST under the control of the oligodendrocyte/ Schwann cell specific PLP-promotor [*J. Neurosci, 2007, 27, 9482].* When brain homogenates of these mice were measured for CST enzymatic activity we found depending on the mouse line up to 200 fold more activity. At the same time, however, the amount of sulfatide in these animals was only slightly increased. Although we do not know the exact molecular reason for this discrepancy a likely explanation could be that also *in vivo* overexpressed CST is largely retained in the endoplasmic reticulum and does not reach the Golgi apparatus in which sulfatide is synthesized.
The many experiments we have performed suggest that CST needs another protein or so far unknown subunit to leave the endoplasmic reticulum. When CST is overexpressed only the fraction of enzyme associating with the postulated factor passes ER quality control and is delivered to the Golgi apparatus. This is a well known phenomenon and has also been described for other proteins [*e.g. J Cell Biol. 2009, 184,173*]. Aim of this project is to search for this unknown factor. For this purpose we

have already developed tools but due to lack of funding we were so far not able to perform the necessary experiments. If this project is successful it will facilitate enormously the development of a high throughput assay searching for therapeutically relevant compounds for MLD.

**Experimental approach**

We will use three approaches to identify proteins interacting with CST.

1. We will make use of the transgenic mice which overexpress CST in oligodendrocytes. The CST expressed from the transgene is a fusion protein bearing a HA-tag at the C-terminus. We have also generated large amounts of an anti-HA monoclonal antibody and coupled it to a sepharose matrix. This sepharose column will be used as an immunoaffinity reagent to purify HA tagged CST from brains of transgenic mice. We have already demonstrated the feasibility of this approach by identifying interaction partners of other HA tagged proteins we are interested in. We will enrich ER fractions of the brain of PLP-CST transgenic mice by subcellular fractionation. These fractions will be solubilized with Triton X-100 or other mild detergents in buffers mimicking the intracellular milieu (e.g. 20 mM NaPi pH 7.4, 100mM KCl, 1 mM Mg++, Ca++, Zn+.) The solubilized fractions will be passed over the HA affinity column. All experiments will be done in parallel also with brains of non-transgenic control mice. It is hard to predict how many brains will be necessary to isolate a sufficient amount of proteins. Initially we plan 20 mice in each group. After washing bound proteins will be eluted with an excess of HA peptide, which ensures that only proteins elute which are bound via the HA tag and therefore reduces elution of unspecifically bound proteins. The eluting fractions will be subjected to 1D / 2D gel electrophoresis, protein spots/bands will be isolated, trypsin digested and subjected to MALDI- TOF or ESI-ion-trap mass spectrometry. Alternatively we will digest the fractions without prior gel-electrophoresis and analyze by LC-MS/MS in the ESI ion trap. CDNA's of proteins identified by this approach will be transiently expressed in the already available CHO cells overexpressing sCST-TAG. If these candidate proteins cause exit of the sCST-Tag from the ER the subcellular localization should be altered. In cells transfected with cDNA's of candidate proteins we will examine the localization of sCST-Tag in the Golgi-apparatus via immunofluorescence with antibodies directed against respective marker proteins and possible presence in the media by Western Blot analysis, respectively. This approach seems the most practical and most promising to us. Therefore we will favour this approach and focus on it. Only if this approach is not successful we will proceed to approach 2.
2. Alternatively we will use an expression cloning approach. For this purpose we already have constructed a CST with a HA-tag at the C-terminus and replaced the transmembrane and intracytosolic domain with that of dipeptidylpeptidase IV. It has been published that the latter targets proteins to the plasma membrane. Since CST is a type II membrane protein the C-terminal HA tag of the fusion protein is luminal in the ER and after transport to the plasma membrane faces the extracellular space. Here the HA tag can be detected by immunofluorescence using an anti-HA antibody on non-permeabilized cells. We will generate CHO cells which express this construct stably. In such cells the construct will largely remain in the ER. IF such a cell is transfected with a plasmid expressing a protein which supports the exit of CST from the ER, one can expect that some of the construct will appear at the plasma membrane and expose the HA Tag on the cell surface. Such cell can de identified by immunofluorescent antibody staining. Millions of cells can be screened visually in a microscope within a reasonable time period. Therefore this procedure is suitable for expression cloning. We will generate a brain cDNA expression library and transfect this library into the CST overexpressing cells. We will then search via anti-HA immunofluorescence on non-permeabilized cells for cells presenting the CST-HA protein at the plasma membrane. Cells in which CST localizes to the plasma membrane are likely to express candidate proteins promoting exit of CST from the ER.
3. If these two techniques yield no results we will switch to a yeast two-hybrid screen. Since in our experience exclusion of false negatives in yeast two hybrid can be very laborious we prefer to pursue approaches 1 and 2 before switching to yeast two hybrid systems.

**Timing**

The techniques using the transgenic mice and the HA affinity column will take about one year. This technique certainly needs multiple modifications before results appear reliable. The Ph. Student will focus on this in the first year. Whenever possible this student will in parallel generate the brain expression library for the expression cloning approach. Only if technique 1 is not successful after one year we will switch to the expression cloning approach will cover year two of the application. If an

interaction partner of CST is found already in the first year the project will focus on the optimization of CST expression using the protein. Yeast two hybrid screens will be started if this is not successful. Most likely this cannot be finished within the two years and is beyond the scope of this proposal.

**Expected Results**

We expect to isolate a protein which promotes the exit of CST from the ER. This will most likely enable us to produce sufficient amounts of secretory CST for screening purposes. It should not be ignored that this project bears the risk of failure. It cannot definitely predicted whether this goal can be achieved. The applicant would like to emphasize that parallel to this project the group will also work on the so far not successful expression bacteria and yeast. This part is funded by the German Ministry of Science (see below). Since substrate reduction therapy holds promise for MLD the applicant believes that a parallel approach is justified to enhance chances for a rapid solution of the problem. Main focus of the funded project is, however, the development of assays detecting cerebroside sulfotransferase activity for high throughput screening.

**Budget**

Total: 115.000 € for two years.

**Justification of Budget**

A) Staff

All techniques which are needed to perform this project are well established in the laboratory of the applicant. Therefore the project does not need an experienced post doc but can be performed by a Ph.D student. The work does at least need two years. A Ph. D student costs about 30.000 € per year, thus a total of 60.000 € in two years

B) Consumables per year

| | |
|---|---|
| Tissue culture media | 3.000 € |
| Plastic ware | 3.000 € |
| Chemicals/Plastic for subcellular Fractionation (e.g.Percoll, Ultracentrifuge Tubes) | 2.000 € |
| Primary and secondary antibodies (Golgi / ER marker / Labelled sec. Antibodies) | 2.000 € |
| Gels for 2 D resolution of purified proteins | 2.500 € |
| Materials for Mass-Spectrometry | 3.000 € |
| Transfection reagents | 2.000 € |
| Materials for library generation | 2.500 € |
| General chemicals | 3.000 € |
| Sum consumables | 23.000 € |

C) Animal housing

Housing is 0.60 € per mouse / per week. It is difficult to predict how many mice we need per experiment to be able to isolate sufficient amounts of CST-HA and associated proteins. If we assume that brains of 20 transgenic mice and 20 control mice are needed per experiment and that one experiment takes place every week for a total of 30 weeks, a total of 600 CST transgenic mice and 600 controls is needed. On average these mice will be 8 weeks of age at time point of sacrifice.
1200 mice X 8 weeks X 0.60 = 5.760 €

In order to breed these mice from our experience it needs 30 breeding pairs which must be maintained for about 40 weeks.
60 mice X 40 weeks X 0.60 € = 1.440 € Total animal cost is ~ 7.200 €

D) Travel. 1.800 € are needed to allow the Ph.D student the visit of revelant meetings

**Qualification for the principal investigator for the project**

The applicant has a longstanding research interest in MLD. He has cloned the ASA gene, revealed genotype-phenotype correlations, constructed various mouse models and performed preclinical therapeutic enzyme replacement studies which paved the way for the ongoing clinical phase I/II studies. His laboratory has all the equipment including mass spectrometers to perform the suggested experiments. All techniques needed are well established in the group.

**Five selected publications of the principal investigator since 2004**

1. U. Matzner, E. Herbst, K. K. Hedayati, R. Lullmann-Rauch, C. Wessig, S. Schroder, C. Eistrup, C. Moller, J. Fogh, V. Gieselmann: Enzyme replacement improves nervous system pathology and function in a mouse model for metachromatic leukodystrophy. *Hum Mol Genet*. 14, 1139-1152 (2005)

2. M. Eckhardt, K.K. Hedayati, J. Pitsch, R. Lüllmann-Rauch, H. Beck, S.N. Fewou, V. Gieselmann: Sulfatide storage in neurons causes hyperexcitability and axonal degeneration in a mouse model of matachromatic leukodystrophy. *J. Neurosci.* 27(34), 9009-21 (2007)

3. H. Ramakrishnan, KK. Hedayati, R. Lüllmann-Rauch, C. Wessig, S.N. Fewou, H. Maier, H.H. Goebel, V. Gieselmann, M. Eckhardt: Increasing sulfatide synthesis in myelin-forming cells of arylsulfatase A-deficient mice causes demyelination and neurological symptoms reminiscent of human metachromatic leukodystrophy. *J. Neurosci,* 27(35), 9482-90 (2007)

4. U. Matzner, F. Matthes, C. Weigelt, C. Andersson, C. Eistrup, J. Fogh, V. Gieselmann: Non-inhibitory antibodies impede lysosomal storage reduction during enzyme replacement therapy of a lysosomal storage disease. *J Mol Med* 86, 433-42 (2008)

5. I. Zöller, M. Meixner, D. Hartmann, H. Büssow, R. Meyer, V. Gieselmann, M. Eckhardt Absence of 2-hydroxylated sphingolipids is compatible with normal neural development but causes late-onset axon and myelin sheath degeneration. *J Neurosci*. 24, 9741-54 (2008)

**Demarcation from other currently funded projects of the applicant**

1. Funding source: DFG (German Research Council)
   Title: Role of 2-hydroxylated sphingolipids in the nervous system and skin

This project is only concerned with the elucidation of hydroxylation of fatty acid residues primarily in myelin of the nervous system.

2. Funding source: ELA (European Leukodystrophy Foundation)
   Title: Molecular mechanisms in the pathophysiology of metachromatic leukodystrophy

This project only covers basic research on MLD. It does not involve any work on CST or therapeutic approaches

3. Funding source: BMBF (German Ministry of Science)
   Title: Metachromatic Leukodystrophy: Strategies for substrate reduction therapy and inverstigations on neuronal and myelin pathology in new mouse models.

This project has two aspects. One investigates alterations in myelin protein and lipid composition in MLD mouse models and tries to reveal how much neuronal storage influences the phenotype in these mouse models. There is no overlap with this application.
The second part is directly related to this application, however, there is also no overlap.
This project develops enzymatic assays to allow large scale determination of CST enzyme activity and thus complements the efforts here. Part of the project also covers attempts to modify CST in a way that may allow expression of the enzyme in bacteria or yeast, which has so far also proven to be impossible. Thus, both projects have the same goal but use different strategies. The urgent need for a therapy justifies parallel strategies to rapidly develop tools for high throughput screening.

**Declaration of applicant:**
This proposal is not funded by other sources and has not been submitted elsewhere

**Project title**

## NEURAL STEM CELL THERAPY FOR MLD

**Principal Investigators**
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**Background**
Arylsulfatase A (ASA) malfunction causes progressive accumulation of intermediate degradation products, leading to cell damage in several organs. The Enzyme Replacement Therapy (ERT) can reverse disease progression in peripheral organs, but not in the CNS, as ASA is unable to cross the blood-brain barrier. A widespread integration of healthy or genetically corrected NSCs inside the MLD brain could correct the enzymatic defect, limiting the lethal progression of the neurodegeneration.

**State of research and own previous work**

In 1999 neural stem cells (NSC) have been identified and isolated from the SVZ of adult mammalian brain. Since that time, many studies have been focused on NSC as a therapeutic tool for neurodegenerative diseases encompassing either genetic diseases like MLD, HD, AD (sporadic) either idiopatic diseases like PD, AD, MS, ALS, stroke etc. NSCs are multipotential precursors that grow and self-renew for extensive time in culture as neurospheres, while retaining a stable capacity to generate mature, functional brain cells for neural repair in neurodegenerative disorders. The major aim of the project is to couple the experience of Dr. Scarpa's clinical and research activities on LSDs and Dr. Vescovi expertise on neural stem cells to investigate the expression of ASA in animal models affected by MLD and receiving intratechal or intracerebroventricular injection of neural stem cells.
Vescovi's lab has been among the first to establish *in vitro* conditions to isolate and propagate NSCs from adult rodent and human fetal brain (Gritti et al., 1995; Gritti et al., 1996; Gritti et al., 1999; Vescovi et al., 1999; Gritti et al., 2002) and from brain tumors (Galli et al., 2004). Upon growth factor removal, NSC cease proliferation and terminally differentiate into the three major neural lineages, astrocytes, oligodendrocytes and functional neurons, able to elicit action potential *in vitro*, as demonstrated by using molecular biology, biochemistry, immunocytochemistry, fluorescence microscopy and electrophysiological assays (Vescovi et al., 1999, De Filippis et al., 2007).
With the aims of using NSCs for therapeutic purposes we have extensively characterized their biological properties *in vitro* and after transplant into different animal models, demonstrating that NSCs are able to integrate within the brain parenchyma without signs of tumorigenity or overgrowth, to migrate inside the CNS into damaged regions and to provide support to degenerating cells. Indeed, our cell lines have been successfully used to develop cell therapy in experimental models of multiple sclerosis (MS) where neural stem cells injected etheir intraveinously or intracerebrally can selectively reach brain and spinal cord areas affected by the demyelinating-inflammatory process and repair and contribute to myelin restoration in those damaged areas. Efficacy is maximal when cells are injected soon after disease start (Pluchino et al., 2003)
We also exploited lentiviral vectors technology to transduce NSC *in vitro* and *in vivo* (Consiglio et al., 2004), without impairing their basic stem cells properties. Indeed, when lentiviral vector carrying the *gfp* gene was injected into the SVZ of adult mice, endogenous stem cells were monitored along the RMS up to the olfactory bulb (OB) and within 6 months from injection a chimeric mosaicism of transduced and not transduced cells was detectable in the OB, demonstrating that neurogenic ability of adult NSC was not affected by lentiviral transduction. These results suggest that this approach can be used to perform genetic trans-correction by cells engineered to induce stable expression of therapeutic genes. This project will be aimed to exploit human NSC for he study and the therapy of metachromatic leucodystrophy (MLD), originating results which could be nonetheless useful to elucidate the possible application of NSC as a tool for the therapy of lysosomal storage diseases (LSDs) in general.
MLD is a genetic disease where the misregulation of a metabolic pathway is caused by the deficit of ASA enzyme which is usually secreted by the producer cells and uptaken by neighbors.
Previous studies from Kawabata et al. (2006) have already shown that neural progenitor cells derived from the striatum of E14 embryo MLD knockout mice and transduced with HIV-ASA vector were able to enhance sulfatide clearance after transplantation into the brain parenchima of adult MLD mice.
Recently, Dr. Vescovi published in collaboration with the lab of E. Bongarzone that presymptomatic

MLD pups showed an improved arylsulfatase (ASA) activity with a significant amelioration of neurodegeneration and motor-learning/memory deficits upon transplantation of dissociated neurospheres from syngenic wild-type animals into the brain of MLD mice. Most of transplanted NSC differentiated spontaneously into the astroglial phenotype able to intrinsically produce ASA enzyme. Indeed, the therapeutic effect of neural stem cells *in vivo* was not due to a replacement of degenerated oligodendrocytes as expected, but to an enzyme-correction from the transplanted to the host cells. This result is in line with the more advancing trend of studies suggesting NSCs as a source of trophic factors, supporting regenerative and neurogenetic processes, besides as a pool for cell replacement therapy.
Altogether the previous results showed that NSC are able to *in vivo* integrate, to migrate and to cross correct a genetic defect either by intrinsic expression of the target protein or by induced expression after genetic manipulation *in vitro* or directly *in vivo*. Relying on these results, in our study we propose to develop a human NSCs mediated therapy in the animal model for MLD disease and improving the strategy by combining NSCs abilities to interact with damaged brain together with lentiviral delivery of wild type enzyme by transplantation of human NSCs (hNSCs) overexpressing the wild type enzyme. Taking advance of the previous results obtained by Kawabata with embryo-murine NSC and by Givogri et al. (2008) with murine adult NSC , we here propose to exploit therapeutic potential of human NSC lines from the telencephalic-diencephalic area of fetal brain which currently are expanded and stored as a GMP biobank for the future clinical trials on patients. The fundamental importance of this project stands in the translational approach which will allow to use neural progenitors derived from highly standardized human NSC lines of wild-type donors as a source for the reconstitution of ASA enzyme activity in MLD mice, either by endogenous ASA expression in hNSC either by lentiviral vector-induced ASA overexpression. Both these strategies will be tested in MLD mice which has been shown a reliable model for MLD therapeutic studies (Kawabata et al., 2006; Givogri et al., 2008). Hopefully, these studies will provide results preliminary to the proposal of GMP grade hNSC lines as a therapeutic tool for future clinical trials on MLD patients.
Experimental plan:
MLD animal models will be injected with human neural stem cells, the grafting and expression of cells will be followed by molecular and biochemical technologies. A major study will be performed to determine the migration and differentiation of the transferred cells at short and long term in order to plane a phase I clinical trial on MLD patients. Several clonal, genetically homogeneous HNSC cell lines have been obtained and are currently generated in GMP grade culture conditions. These human NSCs (hNSCs) do provide a plentiful and renewable source of brain cells for cell-based therapeutic purposes. Therefore, the experiments in this project will be performed with hNSC as preliminary to the clinical trials on patients.

**AIMS OF THE PROJECT**
The principal aim of the project is to investigate about the capability of human neural stem cells to correct the MLD phenotype in vivo, either by replacing impaired oligodendrocytes, either by the cross-correction of ASA deficiency, spontaneously or upon manipulation with therapeutic viral vectors.
We propose the presymptomatic treatment of MLD mice by hNSC therapeutic strategies in order to prevent the progression of the neurological impairment.
The rationale for this task is the accomplishment of the integration of large numbers of new, wild-type or genetically engineered, human neural cells in the diseased brain of MLD mice. This will be achieved as follows:
**Transplantation of wild-type and genetically engineered NSCs**
Several studies have already shown that intracranial (Lee et al., 2007) or intracerebroventricular transplantation (Givogri et al., 2008) of NSC in presymptomatic pups of LSDs mice results in a delayed disease onset, reduced pathology and prolonged survival.
hNSCs cultured in our lab originate from the telencephalic-diencephalic region of fetal human brain from abortive therapeutic fetuses and are grown in the presence of growth factors EGF and FGF2 as neurospheres (Gritti et al., 1999, Vescovi et al., 1999). As the original causes of the neurological impairment in MLD are basically unknown, we propose to transplant two different populations of cells:
a) dissociated neurospheres (NS) as a heterogeneous pool of stem cells, progenitors and differentiated cells.
b) progenitors derived from pre-differentiated NSC (3 days in FGF2 culture medium) .
NSC stable profile with regard to self-renewal, expansion, differentiation and growth factor-dependence has already been confirmed by a well-defined assay platform (Foroni et al., 2007). This work will show that under our culturing conditions, NSC satisfy the most stringent requirements to qualify as a plentiful and safe source of neural cells. We then plan to determine how hNSCs respond in a model of MLD and wether both the populations derived from hNSC (as described above) are equally efficacious. In parallel, we will study whether hNSC could be used in a collaborative and

synergistic manner with another strategy, as transduction with therapeutic lentiviral vectors. As already shown by Givogri et L. (2008), it seems that transplantation of hNSC in presymptomatic animals is quite more effective than in acclaimed symptomatic mice, Thus, we propose to transplant hNSC-derived neurospheres or progenitors in MLD animals at a presymptomatic stage of the disease. We already have shown in previous studies that ICV of NSC succeeds in NSC integration into the SVZ and migration throughout the brain parenchyma in EAE models for multiple sclerosis, as the chronic inflammatory condition generates a permissive environment for the NSC passage through the ependymal layer. As mostly LSDs are characterized by a prominent injurious inflammatory signature (Wada et al., 2000; Myerowitz et al., 2002; Jeyakumar et al., 2003), preliminary experiments will be aimed at the setting of optimal transplantation conditions: either intraparenchimal and intracerebroventricular injection protocols will be tested and evaluated for the engraftment ability of the cells. The efficacy of these approaches will be evaluated through the analysis of the restoration of deficient enzyme activity, reduction of microgliosis and cell vacuolation, neuronal loss, dendritic alterations and synaptic disfunction - in conjunction with grafted cell survival, integration, migration and differentiation. The amount of recombinant ASA released by the cells is strictly dependent on their ability to produce the protein.
Preliminary experiments in vitro will assay the ability of normal and transduced cells to produce the ASA enzyme and trans-correct defective cells. However, such evaluation cannot be predictive of the in vivo results. Once cells have been injected or implanted, the recombinant ASA needs to be taken up by the endogenous defective cells, through the mannose-6-phosphate receptors. Induced recombinant enzyme activity can be easily monitored through a fluorimetric assay while its effectiveness can be assessed by measuring tissue SULFATIDES contents, which will be evaluated both biochemically and histochemically.

**Timing**

The project will be developed in two years time.
**First year**:
0-7months:
a. One or two broods obtained by MLD mice will be devoted to test the efficiency of hNSC delivery through intratechal and intracerebroventricular injection into the brain.
b. Expansion of hNSC lines, infection with lentiviral vectors lenti-RFP and lenti-RFP/ASA. Analysis of expression of ASA either intrinsically by lenti-RFP transduced hNSC, either enhanced by lenti-RFP/ASA hNSCs.

7-12 months:
Once the pups will be transplanted (birth, P0), their brains will be analyzed 14 days post transplantation. Animals will be sacrificed and their brains will be processed for immunohistological analysis to detect the distribution of RFP. The analysis will provide a quantitative and qualitative evaluation of the diffusion of NSC from the cortex to the deeper layers of the brain for intratechal injection or from the SVZ through the striatum and finally to the upper layers of the brain for intracerebrovetricular injection.
Before paraformaldheide fixation, half of the brain will be processed to perform biochemical and histochemical determination of cerebroside-3-sulfate (sulfatide) content and evaluation of induced ASA enzymatic activity.
**Second year**:
Months 0-12:
a. After the selection of the transplantation way of delivery for hNSC, homogeneous broods of pups will be transplanted. In parallel, wild-type syngenic animals will be transplanted.
b. Transplanted animals will be analyzed for the recovery of behavioural performances .
Months 3-12:
Immunohistochemical and biochemical analysis of brains

**Expected Results**
We expect to elucidate the capability of neural stem cells to correct the MLD phenotype in vivo, either by replacing impaired oligodendocytes, or by the cross-correction of ASA deficiency, spontaneously or upon manipulation with therapeutic viral vectors.
**Budget**
Total: **60.000 € for two years (UNimib)**.

**Justification of Budget**
Staff:
35.000euro for the fellowship of a post-doc for the help in cell culture , lentiviral infection, immunohistochemistry , transplantation and analysis of the data (6 months per year, 17.500 euro per year)
4000 euro for the construction and the generation of the lentiviral vectors carrying control reporter genes or ASA gene.
14.000 euros for disposable material (pipettes, tips, flasks for cell cultures, dishes, basic components of thye culture medium,chemical reagents and antibodies)
3000 euros for trips to Padova for transplantation or for meetings aimed to the analysis of the data
4000 euros for animal facility (MLD mice will be host for analysis in vivo or for primary cultures from the brain)

**Qualification for the principal investigator for the project**
Angelo Vescovi is Associate Professor at the Department of Biotechnology and Biosciences of the University of Milan-Bicocca. Following the BTBS mission, Prof. Vescovi has recently committed himself to the creation of consortiums and companies in order to efficiently link the University, the local institutions and industry to perform cutting-edge scientific research. His projects throughout the years have been funded by national and international granting agencies and his research group has a long lasting history of collaboration with national and international research institutes and biotech companies. His research projects have mainly focused on the study of neural stem cells derived from fetal and adult tissue, and their extensive characterization and use in different animal models of neurodegenerative diseases and brain cancer.
The group directed by Prof. Vescovi has extensive experience in the establishment of neural stem cell lines from adult murine and human brain. Covering all aspects from their molecular and functional characterization: analysis of proliferation, differentiation assay, cytofluorimetric analysis of the immunophenotype and transplantation in animal models of neurodegenerative diseases.

Five selected publications of the principal investigator since 2007

-Consiglio A, Gritti A, Dolcetta D, Follenzi A, Bordignon C, Gage FH, Vescovi AL, Naldini L. 2004. Robust in vivo gene transfer into adult mammalian neural stem cells by lentiviral vectors. Proc Natl.Acad Sci U S A 101:14835-14840.

-Givogri MI, Bottai D, Zhu HL, Fasano S, Lamorte G, Brambilla R, Vescovi A, Wrabetz L, Bongarzone E. 2008. Multipotential neural precursors transplanted into the metachromatic leukodystrophy brain fail to generate oligodendrocytes but contribute to limit brain dysfunction. Dev Neurosci 30:340-357.

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-Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R. 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156:71-83.

--De Filippis L., Lamorte Giuseppe , Snyder E.Y., Malgaroli A. & Vescovi A.L.. A novel, immortal, multipotent human neural stem cell line, generatine functional neurone and oligodendrocytes. Stem Cells, 2007.

**Budget**
Total: **60.000 € for two years (UNIPD)**.

**Justification of Budget**
Staff:
35.000euro for the fellowship of a post-doc for animal manipulation and transplantation of neural stem cells, analysis of expression after transplantation of the transduced ASA and microarray analysis of transduced and control animals.

20.000 euros for disposable material (pipettes, tips, flasks for cell cultures, dishes, basic components of thye culture medium,chemical reagents and antibodies)
5000 euros for animal facility

**Qualification for the principal investigator for the project**

Maurizio Scarpa is Assistant Professor of Pediatrics at the Department of Pediatrics of the University of Padova. He is leading the Unit of Lysosomal Storage Diseases, a major referral centre in Italy on these disorders. He is collaborating with the major Biotech Companies (Actelion, Genzyme, SHIRE HGT and BIOMARIN) to draw guidelines for the treatment and the management of patients affected by LSDs and to optimize the efficiency and safety of recombinant drugs. He is the chairman of the European CNS Working Group in SHIRE HGT.
The group lead by MS in focused at the development of new strategies to deliver recombinant enzymes by nanoparticles and gene therapy tools. Recently the group is studying the effect of storage on the Blood Brain Barrier of MPS animal models in Collaboration with David Begley, Kings College of London with whom he had funded the Brains for Brain European Reasearch Consortium.
He is the Vice president of the Brains For Brain Foundation.

Five selected publications of the principal investigator since 2007

-Gasparotto N, Tomanin R, Frigo AC, Niizawa G, Pasquini E, Blanco M, Donati MA, Keutzer J, Zacchello F, Scarpa M. Rapid diagnostic testing procedures for lysosomal storage disorders: alpha-glucosidase and beta-galactosidase assays on dried blood spots.,
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-Friso A, Tomanin R, Zanetti A, Mennuni C, Calvaruso F, La Monica N, Marin O, Zacchello F, Scarpa M. Gene therapy of Hunter syndrome: evaluation of the efficiency of muscle electro gene transfer for the production and release of recombinant iduronate-2-sulfatase (IDS).
Biochim Biophys Acta. 2008 Oct;1782(10):574-80.

-Begley Dj, Pontikis Cc, Scarpa M. Lysosomal storage diseases and the blood-brain barrier.
Curr.Pharm. Des, 2008 vol. 14, p. 1566-1580,

-Grisafi D, Piccoli M, Pozzobon M, Ditadi A, Zaramella P, Chiandetti L, Zanon, Gf, Atala A, Zacchello F, Scarpa M., De Coppi P, Tomanin R (2008). High transduction efficiency of human amniotic fluid stem cells mediated by adenovirus vectors
Stem Cells Dev. 2008 Oct;17(5):953-62.

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Eur J Pediatr. 2008 Mar;167(3):267-77

**Project Title:**

**Hematopoietic stem cell gene therapy for the treatment of metachromatic leukodystrophy**

**Principal Investigator:**
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**Background, significance and state of research**

Our group is intensively working on the development of a gene therapy strategy for the treatment of Metachromatic Leukodystrophy (MLD) based on the transplantation of autologous hematopoietic stem and progenitor cells (HSC) transduced with Lentiviral Vectors (LV) encoding the functional Arylsulfatase A (ARSA) cDNA [1,2]. HSC gene therapy has long been considered an attractive option for the treatment of Lysosomal Storage Disorders (LSD), and in particular for Nervous System (NS) manifestations. Based on pre-clinical and clinical experience of HSC transplantation (HSCT), we might expect autologous HSC gene therapy to be able to: i) exploit the unique properties of the HSCT, capable of repopulating affected tissues with donor-derived myeloid cells, which then become an effective tissue source of the functional enzyme [3]; ii) improve the therapeutic potential of HSCT, since autologous cells may be genetically-modified to constitutively express higher levels of the therapeutic enzyme and become a quantitatively more effective source of enzyme than wild-type cells, possibly also at the level of the NS [2]; iii) reduce allogeneic HSCT side effects, since the autologous procedure is expected to be associated to a reduced transplant-related morbidity and mortality, and avoids the risks of Graft versus Host Disease (GVHD). Therapeutic efficacy of HSC gene therapy in controlling disease manifestations has been shown in preclinical experiments on LSD models [4,5]. In the case of MLD, early studies of HSC gene therapy using retroviral vectors (RV) had a relatively unsatisfactory outcome in the mouse model probably because of the limited HSC transduction rate and low enzyme expression levels in the myeloid extra-vascular progeny by RV [6,7]. These results, together with the poor outcome of allogeneic HSCT in MLD patients, highlight a likely requirement for high levels of ARSA to correct the metabolic defect in the Central NS (CNS). The requirement for high-frequency HSC transduction and for robust sustained levels of therapeutic gene expression in their progeny, and the lack of selective growth-advantage for the corrected cells in MLD, render LV prime candidates for HSC gene transfer in MLD gene therapy. LV integrate efficiently into a variety of cell types ex vivo and in vivo and allow stable and robust transgene expression in the transduced cells and in their progeny. Because of these features, LV enable efficient gene marking of mouse and human HSC with minimal in vitro manipulation and cell perturbation, allowing for full maintenance of stem cell properties and multiclonal repopulation of chimeric hosts [8-11]. Indeed, our work demonstrated that ARSA over-expression by LV in HSC and their progeny allows attaining therapeutic efficacy by HSC gene therapy in the MLD mouse model, both in terms of prevention [1] and correction of neurologic disease manifestations [2]. Importantly, we showed that the degree of efficacy of gene therapy is dependent on the levels of enzyme activity in HSC and in target organs, thus strengthening the concept that the unique advantage of gene therapy over allogeneic HSC transplantation might stem on the possibility of expressing the functional enzyme in HSC and in their progeny largely above normal donors' levels. Thus, our preclinical data indicate that ARSA over-expression is the fundamental therapeutic mechanism to achieve prevention of MLD manifestations and even correction of already established neurological disease. Moreover, we showed that the extent of ARSA reconstitution in treated mice is dependent on the engraftment level and vector dose in transduced HSC, and highlighted a requirement for enzyme over-expression in the transplanted HSC and their progeny in order to overcome the limited benefit of wild type cell transplantation. This can be achieved by combining myeloablative conditioning to allow high-level engraftment of transplanted cells and high-frequency transduction of HSC.

Since ARSA over-expression relies on multiple LV integrations into the transduced HSC, the consequences of multiple LV integrations into the genome should be evaluated. Although the superior proficiency of LV at integrating into different cell types provides a critical advantage over other vectors for gene therapy applications, it may also imply an increased risk of insertional mutagenesis[12]. In a stringent preclinical murine transplant model, we demonstrated that integration of a prototypical self inactivating (SIN) LV has low genotoxic potential [13,14]. Our model showed that the risk associated with insertional mutagenesis is significantly lower for the LV proposed for this application than for conventional RV currently in clinical use. This data, together with the high gene transfer efficiency and therapeutic efficacy demonstrated in preclinical models, provides a major scientific rationale for advancing LV to clinical experimentation.

In the perspective of a clinical application of gene therapy for the treatment of MLD, it was of great importance to address the efficacy and safety of ARSA over-expression into target cells, namely human HSC. We performed an extensive validation of the safety and efficacy of ARSA over-expression in human HSC. In particular, the possibility to achieve ARSA over-expression in the transduced HSC and in their progeny, as well as the safety of over-expression have been demonstrated in relevant murine and hematochimeric models [15]. Moreover, we demonstrated the long-term in vivo persistence of ARSA over-expression in human hematopoietic cells in

hematochimeric models. Further, the metabolic consequences of ARSA over-expression were analyzed in depth. Sulfatase Modifying Factor 1 (SUMF1), encoding for a formylglycine-generating enzyme, has been recently identified as common activator of sulfatases and essential factor for their activity [16,17]. Since it has been suggested that SUMF1 might be a rate-limiting factor in the biological activation of these enzymes, we showed that the over-expression of one sulfatase (ARSA) does not lead to reduced activity of other sulfatases by a competitive interaction with their common activator[15].

The transduction of CD34+ cells from human bone marrow (BM) was then optimized with LV produced in pre-GMP and GMP conditions, with the aim of achieving high transduction rate, a reproducible and upper-limited level of vector content, clinically applicable timing, and optimal maintenance of cell viability and functional properties. A clinically applicable protocol based on 24 hours pre-stimulation and two rounds of transduction was optimized. This protocol, when applied in small or large scale, reproducibly allowed sustained transduction of hematopoietic progenitors and stem cells. When MLD patients' HSC were transduced, enzymatic activity reconstitution and up to 15 fold ARSA over-expression above basal levels was observed. The transduction protocol did not alter the clonogenic potential of the transduced cells, nor their long-term repopulation and differentiation ability in chimeric models. The cells efficiently repopulated long-term the bone marrow, spleen and thymus of recipient mice and differentiated in myeloid, B and T cells; thymic maturation of human T lymphocytes was observed. Molecular analysis on repopulated chimeras confirmed the VCN observed in pre-transplant samples. We also studied the pattern of vector integration on pre-transplant cells and tissues from long-term repopulated mice. Thus far we identified more than 2000 unique integration sites. The vector distribution with respect to the genomic features was similar to the previously described one for LV. Gene ontology analysis did not reveal any bias for gene classes involved in cancer or cell proliferation. Moreover, no skewing in genomic distribution or specific gene classes was detected by comparing genes targeted in pre- and post-transplant samples (Biffi, Montini et al., submitted manuscript).

According to this work, the medicinal product "Autologous CD34+ cells transfected with lentiviral vector containing the human arylsulfatase A cDNA for treatment of metachromatic leukodystrophy" received the Orphan Drug Status by the European Commission of Orphan Drug Designation (Orphan Drug Status EMEA/COMP 84536/2007). We are now implementing a clinical trial of HSC gene therapy for the treatment of a small cohort of MLD patients.

**Research plan**

A protocol based on myeloablative conditioning followed by transplantation of autologous, LV-transduced BM CD34+ cells has been designed. Large scale, GMP grade LV encoding the functional ARSA cDNA have been produced by MolMed S.p.A. and are in the process of being completely characterized for release and clinical use. The trial will be conducted within the Pediatric Clinical Research Unit of HSR-TIGET, which is part of the Bone Marrow Transplantation Unit at HSR. Based on actual patients' accrual and the available vector stocks we plan to treat a total of 8 MLD patients in the first 3 years of the trial. Patients' selection and monitoring will be performed according to validated clinical and instrumental read-outs [18].

A IND for trial approval will be presented to the Italian Regulatory Authorities and to the Ethical Committee of our Hospital by late summer/autumn 2009, as soon as the GMP vector stock will be released by MolMed S.p.A.. Therefore, we expect to begin patients' recruitment by the end of 2009, thanks to our clinical activity and external collaborations.

***In the context of this clinical trial, the goal of the proposed project is the assessment of the biological efficacy and safety of LV-based HSC gene therapy in MLD patients.***

***Biological efficacy*** will consist of:

- long-term engraftment and multi-lineage differentiation of the transduced HSC, which will be assessed by quantitative PCR on peripheral blood mononuclear cells (PBMC) and BM – total and sorted population - from transplanted patients at regular intervals; BM cells will also be plated for clonogenic assays in order to quantify the overall engraftment of transduced cells and vector content in clones, and transplanted into immunodeficient mice to perform in vivo clonal tracking studies;
- long-term ARSA activity reconstitution and enzyme over-expression in the hematopoietic system: ARSA mRNA production will be monitored and quantified on pre- and post-transplant samples (by quantitative PCR) and ARSA activity will be measured on post-transplant PBMC and BM samples using patients' pre-transplant un-transduced samples and normal controls as reference; if enough material will be available, other sulfatases activity will be tested.
- ARSA delivery to the CNS and PNS by the progeny of the transplanted HSC, which will be assessed by ARSA activity measurement on the cerebrospinal fluid and on skin biopsies (containing small myelinated fibers) from treated patients, and by sulfatide TLC quantification on the same specimens. Demyelination and storage (metachromatic granules) within white matter fibers will be also monitored on skin biopsies.

These results will be integrated with the data coming from the clinical and instrumental follow up of the treated patients in order to correlate biological efficacy to clinical benefit.

Correlation between the level of ARSA activity measured in PBMC and/or BM cells and clinical phenotype in untreated MLD patients or in patients undergoing allogenic BM transplantation has not been reported. In this unique and novel setting potentially allowing the achievement of enzyme over-expression in the hematopoietic system and partial reconstitution of ARSA activity in the NS, we will attempt establishing a correlation between transduced cell engraftment, ARSA activity and clinical benefit in gene therapy treated patients.

***Safety*** of HSC gene therapy will consist of:

- the absence of recombination-competent LV (RCL), assessed by p24 test on patients' serum, and by PCR amplifying HIV mRNA and VSV DNA;
- the absence of abnormal clonal proliferation, assessed by monitoring complete blood count, protein serum electrophoresis, flow cytometry on PBMC, TCR Vbeta families, vector copy number in cells, bone marrow karyotype, immunophenotype and morphology;
- absence of immune responses against the transgene;
- integration studies on pre- and post-transplant samples will be performed, as part of the overall assessment of LV gene transfer safety, on transduced pre-transplant patients' HSC samples and on PBMC and BM of transplanted patients once achieved stable transduced cells engraftment. These studies will allow assessing the distribution of LV-ARSA integration sites according to the type of genomic elements, detecting any similarities or discrepancies with the pre-clinical studies and the other on going gene therapy trials. Further, we will monitor the repopulation clonality over time, the long-term persistence and stability of transduced cells engraftment and the occurrence of in vivo skewing in the distribution of vector integration sites, as compared to the pre-transplant samples.

**Timing**

The trial will begin by the end of 2009 and 2/3 patients per year will be enrolled. Relevant information will be obtained starting from the first relevant patients' follow up at 1 year after treatment. Therefore, data interpretation will require a minimum of 2 years of work to be initiated.

**Expected results**

The MLD patients undergoing HSC gene therapy will represent a unique model to study engraftment, long-term survival and differentiation of transduced HSC. Therefore, as mentioned above, we will perform clonal tracking studies in vivo both in patients and in chimeras repopulated with patients' stem cells. Tracking of individual stem and progenitor cell clones in vivo by integration site analysis will allow to define the number of HSC clones contributing to stable hematopoiesis, their self renewal, and lineage commitment. Different hematopoietic cell subsets will be sampled following cell sorting or immunomagnetic enrichment. CD34+ progenitor cells from treated patients will be also plated in clonogenic assays (CFU-C, LTC-IC, B/NK assay) for their capacity to differentiate into multiple lineages. In addition, CD34+ cells will be transplanted in RAG2-/-, IL2R-gamma chain-/- mice, to study repopulation capacity in vivo and allow clonality assessment in progenitor-derived cells. Vector integrations will be cloned from sorted patients' cells as well as from human-mouse hematopoietic chimeras, as described above. RNA will be also purified and stored to permit correlation analysis of gene expression with integrations. The presence of common integrants among different lineages at different time points and in cells obtained from repopulation assays will be considered a formal proof that single HSC clones with multilineage potential stably engrafted in the patients.

## A. Literature Cited

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2.Biffi A, Capotondo A, Fasano S, et al. Gene therapy of metachromatic leukodystrophy reverses neurological damage and deficits in mice. J Clin Invest. 2006;116:3070-3082.

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15.Capotondo A, Cesani M, Pepe S, et al. Safety of Arylsulfatase A Overexpression for Gene Therapy of Metachromatic Leukodystrophy. Hum Gene Ther. 2007;18:821-836.
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**Budget :** Total 80.000 €

**BUDGET JUSTIFICATION**

**Personnel**
*Alessia Capotondo, Post-Doc*, has extensively participated to the development of gene therapy for MLD. She will be involved in the biochemical and molecular follow up of transplanted patients. Given the complexity of her tasks it needs at least 50% effort of her total time to manage the project. This corresponds to 26.000 €.

**Supplies**
Supply costs are conservative estimates based upon actual expenditures in our laboratory over the past years and they reflect the minimum needs to carry on the proposed experiments. In particular the budget has been designed to cover the costs of the follow up of 2 out of the 4-6 patients that will be enrolled in the first 2 years of the trial. Other already available funding (see below) will cover the costs for the other patients' follow up. The costs for vector production, patients' HSC transduction, and patients' treatment and follow up will be covered by the Italian Telethon Foundation, and thus are not included in this Budget. This budget comprises the costs for the biochemical and molecular studies. In particular, the expected cost of the follow up for 1 patient is 25.000€ and includes reagents and supplies for: qPCR for engraftment determination (BM and PBMC, bulk and selected populations); ARSA activity measurement (BM, PBMC, byoptic material); ARSA mRNA qPCR (complete set up and standardization, and BM and PBMC); in vitro clonogenic assays on patients' BM samples; hematochimeric mice generation and follow up (evaluation of human cells engraftment in hematopoietic tissues by FACS analysis); integration studies in pre- and post-transplant samples (expected ≥60 samples). The applicant calculates a total of 50.000 € for supplies.

**Travel**
Domestic and International travel funds are requested to present results at annual meetings of the American Society for Gene Therapy (1 person, estimated cost 3.000€), European Society for Gene Therapy (1 person, estimated cost 1.000€).

**Qualification of the principal investigator for the project**
The applicant has a longstanding research interest in MLD and in the development of a gene therapy approach for MLD treatment. Her studies demonstrated the therapeutic potential of HSC gene therapy in treating LSD with nervous system involvement and identified a new means for targeting therapeutic molecules to the NS. According to this work, a clinical trial of HSC gene therapy in MLD patients will begin in HSR by summer 2009, being A. Biffi co-Principal Investigator of the study (Orphan Drug Status EMEA/COMP 84536/2007). Recently, she started

working on two additional LSD, globoid leukodystrophy and type 1 Mucopolysaccharidosis, with promising preliminary results. She authored 17 scientific papers (total IF: 112.4) of which 9 as first, 2 as last and 7 as corresponding Author. She received two Excellence in Research awards from the American Society of Gene Therapy (2004, 2005) and she serves as reviewer for the Annual Meetings of the society. She was invited as speaker to several international meetings, such as the XIV and XVI Congress of the European Society of Gene Therapy (2007, 2008), the 25th International Congress of Pediatrics (2007). She works as reviewer for Human Gene Therapy, The Journal of Gene Medicine, Neurobiology of Disease, Journal of Neuroscience.

**5 selected publications of the P.I.**


1. Biffi A., Lucchini G., Rovelli A., Sessa M. (2008). Metachromatic leukodystrophy: an overview of current and prospective treatments. Bone Marrow Transpl. 42: S2-6.
2. Biffi A., Cesani M., Fumagalli F., del Carro U., Baldoli C., Canale S., Gerevini S., Amadio S., Falautano M., Rovelli A., Comi G., Roncarolo M.G., Sessa M. (2008). Metachromatic leukodystrophy - mutation analysis provides further evidence of genotype-phenotype correlation. Clin Genet. 74: 349-357.
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**Demarcation from other currently funded projects of the applicant**

| Funding Agency, Grant Title | Research area |
|---|---|
| Telethon TGTB01: "Hematopoietic Stem Cell Gene Therapy for Metachromatic and Globoid Cell Leukodystrophies" | Preclinical studies of HSC gene therapy in MLD and GLD |
| E.L.A.: "Arylsulfatase A over-expression in human hematopoietic cells for MLD gene therapy: pre-clinical and clinical assessment" | Preclinical and clinical safety studies of HSC gene therapy for MLD (will cover the costs for the molecular and biochemical follow up of 2 enrolled patients) |
| Ministero della Sanità: "Toxicity induced by lentiviral-mediated over-expression of galactocerebrosidase in stem cells: implications for the development of effective therapeutic strategies for globoid leukodystrophy" | Development of HSC gene therapy for GLD |
| National Tay Sachs and Allied Diseases: "Evaluation of combined approaches using hematopoietic and neural stem cells for the treatment of globoid cell leukodystrophy" | Development of HSC gene therapy for GLD |
| National Mucopolysaccharidosis Society (NMPS): "Novel efficacious and safe gene therapy approaches for the treatment of type I Mucopolysaccharidosis" | Development of HSC gene therapy for MPS I |