Beneficiary no.	Beneficiary name	Beneficiary Short Name	Country
1 (coordi-nator)	UNIVERSITE D'AUVERGNE CLERMONT FERRAND 1	UDA	France
2	OSPEDALE PEDIATRICO BAMBINO GESU	OPBG	Italy
3	ISTITUTO GIANNINA GASLINI	IGG	Italy
4	UNIVERSITAETSKLINIKUM BONN	UKB	Germany
5	INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE	INSERM	France
6	SOLUSCIENCE SA	Soluscience	France
7	THE UNIVERSITY OF MANCHESTER	UMAN	UK
8	FUNDACIO PRIVADA INSTITUT D'INVESTIGACIO BIOMEDICA DE BELLVITGE	IDIBELL	Spain
9	MAX PLANCK GESELLSCHAFT ZUR FOERDERUNG DER WISSENSCHAFTEN E.V.	MPG	Germany
10	MEDIZINISCHE UNIVERSITAET WIEN	MUW	Austria
11	TROPHOS SA	Trophos	France
12	UNIVERSITY COLLEGE LONDON	UCL	UK
14	FONDAZIONE CENTRO SAN RAFFAELE DEL MONTE TABOR	HSR	Italy
15	ACADEMISCH MEDISCH CENTRUM	AMC	Netherlands
16	UNIVERSITE PARIS DESCARTES	LEM	France
17	FRANCE EUROPE INNOVATION	FEI	France
18	ASSOCIATION EUROPEENNE CONTRE LES LEUCODYSTROPHIES	ELA	France
19	THE CHANCELLOR, MASTERS AND SCHOLARS OF THE UNIVERSITY OF CAMBRIDGE	CMSUC	UK
20	UNIVERSITE PARIS DIDEROT - PARIS 7	UPD-P7	France
21	FONDAZIONE IRCCS ISTITUTO NEUROLOGICO CARLO BESTA	FIINCB	Italy
22	UNIVERSITATSMEDIZIN DER JOHANNES GUTENBERG-UNIVERSITAT MAINZ	MAINZ	Germany
23	UNIVERSITAETSKLINIKUM HAMBURG-EPPENDORF	UKE	Germany
24	EBERHARD KARLS UNIVERSITAET TUEBINGEN	EKUT	Germany
25	UNIVERSITY NEW SOUTH WALES	UNSW	Australia

Executive summary (1 page)

Leukodystrophies (LDs) are inherited rare neurodegenerative diseases of the white matter and its main component, the myelin that are affecting predominantly children. Severity of the disease is related to the axonal dysfunction due to myelin deficiency or destruction. Despite the achievement of remarkable advances made in the past decade, there is no current curative therapy. The development of therapeutic approaches for myelin repair and neuroprotection was the main objective of the FP7-LeukoTreat project (starting date: 01/03/2010 – duration: 42 months). Indeed LDs constitute prototypic pathologies to tackle myelin formation/destruction issues as well as glial cells dysfunctions in neurodegeneration.
The global aim of the project was to promote the development of therapeutic strategies for the largest number of LD affected patients by combining the expertise of (i) 19 recognized European fundamental and clinical research teams and one SME (Trophos) in the field of 7 LDs of known cause; (ii) high-technology SME (Soluscience) in the field of information systems for medical records; (iii) experts in medical ethics (Ethic laboratory of the Paris Descartes University) and (iv) LD patients and families associations (coordinated by the ELA Association). The administrative and financial coordination of the whole project has been performed by the French SME France Europe Innovation.

The LeukoTreat project developed 5 complementary success approaches:

(i) WP1 - **characterizing leukodystrophies for therapies** aims to collect, through a unique European database (named “LeukoDB”), the clinical data, mutations and biological samples of more than 500 patients (original objectives) in order to improve knowledge on the epidemiology, the natural history, the genotype/phenotype correlation of LDs for a sufficient number of patients;

(ii) WP2 - **identifying biomarkers** for LDs in order to validate, optimize and investigate the signification of biomarkers identified in biological fluids or cells of LDs affected patients (N-acetylaspartate/N-acetylaspartylglutamate (NAA/NAAG) and eIF2B activity) and to screen for new biomarkers by investigating the role of the oxidative stress in the axonal degeneration common to different forms of slow progressive LDs, and by applying to LDs an innovative lipidomics analysis;

(iii) WP3 - **developing pharmacological strategies** for prototypic leukodystrophies: by using medium-throughput compound screening system that was applied to a yeast model of eIF2B mutations and by testing particular molecules to lower the toxic overexpression of a gene (in PMD), anti-oxidant, anti-inflammatory, or neuroprotective compounds (in X-ALD, PMD), or enzyme replacement therapy (in MLD) to well-characterized mouse models as a prerequisite for considering subsequent clinical trials.

(iv) WP4 - **developing innovative gene and cell therapies** with the objectives (a) to pursue and interpret the ongoing clinical trials using HSC and LV for ALD and MLD for further application to selected other leukodystrophies (LDs), (b) to unravel the mechanisms of disease correction in the brain by studying microglia reconstitution after transplantation, (c) to test the feasibility and therapeutic potential of alternative approaches: direct gene delivery to the brain by using intracerebral injection of adeno-associated virus vectors (AAV) or LV for Canavan disease and MLD; gene silencing approaches specifically targeting oligodendrocytes for Pelizaeus-Merzbacher disease; Neural stem cells (NSC) or microglia precursor-based approaches to treat LDs.

(v) WP5 - **tackling ethical impacts** of the proposed therapeutic challenges by integrating the participation of patients driven by a well-experienced research team strongly skilled in ethics.

COMPLETE SUMMARY

Project context and main objectives

Leukodystrophies (LDs) are inherited rare neurodegenerative diseases of the white matter and its main component, the myelin that are affecting predominantly children. Severity of the disease is related to the axonal dysfunction due to myelin deficiency or destruction. Despite the achievement of remarkable advances made in the past decade, there is no current curative therapy. The development of therapeutic approaches for myelin repair and neuroprotection was the main objective of the FP7-LeukoTreat project (starting date: 01/03/2010 – duration: 42 months). Indeed LDs constitute prototypic pathologies to tackle myelin formation/destruction issues as well as glial cells dysfunctions in neurodegeneration.
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The LeukoTreat project developed 5 complementary success approaches in 5 WorkPackages (WP):

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(v) WP5 - **tackling ethical impacts** of the proposed therapeutic challenges by integrating the participation of patients driven by a well-experienced research team strongly skilled in ethics.

In details:

WP1 - characterizing leukodystrophies for therapies

LDs being rare disorders, the development of cutting-edge therapies must be considered at a European scale. The collection of patients' clinical data to determine the **natural history** and the **epidemiology** as well as **genotype-phenotype correlations** through Europe represents the first key challenge that the consortium aimed to solve together. Numerous initiatives have been previously established in the field of clinical data and biobank for LDs at a national scale or for a restricted group of LDs at European scale. These ones needed to be coordinated and expanded, it was one of goals of LeukoTreat.

As a reminder, participants of the ENBDD Biomed project, including 6 LeukoTreat partners, have established in 1996 a European protocol to collect clinical data and biological samples for LDs of undetermined origin. Efficacy of this strategy proved to be successful allowing the identification and/or phenotype definition of 9 new causative genes (*GFAP, 5 EIF2B, GJA12, MLC1, Hyccin*) of LDs by LeukoTreat partners during the last decade. This European initiative has also been the first step for the establishment of national networks devoted to LDs particularly in France, Germany and Italy :
- In France, epidemiological studies have been initiated by partner UDA with the support of the French clinical research program based on the French reference centers. More recently, an information system have been elaborated with the support of a European regional innovative actions, e-nnovergne-lifegrid (2006-2008, www.lifegrid.fr) using the technology developed by partner Soluscience. The system integrates data generated by the different diagnostic and research platforms: patient informatics chart, imaging, genomic, transcriptomic, proteomic and biobank. Integrated links with more general, web-based bioinformatics database allows a data mining of the results.
- In Germany, following the previous epidemiological studies on LDs based on biochemical markers, Leukonet pursued and extended this survey. The Leukonet is supported by a central databank containing a basic set of all patients and central, disease-specific databanks containing detailed description of patients.
- In Italy, clinical and research teams involved in LDs have more recently been brought together by the Italian myelin project. Teams in Milano (HSR), Genova (IGG) and Roma (OPBG) have been particularly active. Partner IGG have settled a biobank for rare neurological disorders including LDs.

Based on this existing expertise, LeukoTreat proposed to coordinate (in WP1) the collection and the management of clinical data, biological samples and mutations through a European database, the **LeukoDB**. The ultimate goal is to correctly collect 500 patients at the end of the project in order to achieve relevant epidemiological and genotype/phenotype correlations studies at M42. The organization we proposed to set up was based on the three centers (LeukoFrance, Leukonet and LeukoItaly), that have already developed strong skills/infrastructures to support data/samples management and that will together be connected via an innovative web-based information system. Each of these three Leuko reference centers (LeukoRec) should then stimulate the development of other reference centers in Europe and will achieve their connection to the LeukoTreat system. The LeukoDB will allow a clear gain of knowledge on natural course of the diseases, on their epidemiology and genotype-phenotype correlations. This information is required for the establishment of appropriate therapeutic trials that will be facilitated by the establishment of tools for the generation of patients cohorts and electronic clinical forms.

The main objective of WP1 was thus to generate all the necessary information on patients' clinical data, mutations and biological samples to efficiently set up therapeutic trials. This indispensable information consists in :
- correlating the genotype of patients with their phenotype by linking the mutation data and the clinical data,
- correlating the phenotype of patients with the dosages made on patients' samples (biomarkers),
- evaluating precisely the number of patients in Europe to measure incidence and prevalence of each type of LD,
- providing diagnosis assistance so as to open the knowledge and know-how to every European country,
- improving the management of therapeutic clinical trials through clinical research forms.

To reach these goals, the crucial starting step was to coordinate the collection and the management of clinical data, biological samples and mutations through a European database by interconnecting the preexisting data centers (France, Germany and Italy). This European database will represent the largest database of LD patients: therefore it will allow a clear gain of knowledge on the diagnosis and natural course of the diseases but also on their epidemiology. Ultimately, all this information will strongly support the establishment of appropriate therapeutic trials.

WP2 - Biomarkers for leukodystrophies treatment

The aim of the WP2 was to determine the specificity and significance of biomarkers already identified in individual groups of LDs to establish their relevance for therapeutic decisions or follow up in LDs and to identify new therapeutic targets.
Biomarkers are key tools for monitoring treatments, and also as prognostic factors, for the optimization of therapeutic options. Our main goal was to identify and validate diagnostic and prognostic markers taking benefit of the interactions created through the project to have access to both:
-faithful LD models established by the different partners.
-human samples collected and related to reliable clinical and molecular data through the LeukoDB (WP1).
For this purpose two complementary approaches has been used:
- validate, optimize and investigate the signification of biomarkers identified in biological fluids or cells of LDs affected patients (N-acetylaspartate/N-acetylaspartylglutamate (NAA/NAAG) and eIF2B activity).
- screen for new biomarkers by investigating the role of the oxidative stress in the axonal degeneration common to different forms of slow progressive LDs, and by applying to LDs an innovative lipidomics analysis.

WP3 – Pharmacological strategies to treat leukodystrophies

Our main aim in **WP3** was to define and advance pharmacological therapy concepts for prototypic leukodystrophies. The selected leukodystrophies are distinct with respect to their causative genes and key aspects of the pathophysiology. We have thus followed distinct strategies depending on the current knowledge about the particular diseases. For two selected leukodystrophies, CACH and AXD, candidate compounds were not obvious despite considerable knowledge about the respective pathophysiology. In these cases, novel systems had to be established for the screening of the efficacy of compounds that, upon their application to the relevant cellular or mouse models, could enhance the cellular synthesis of proteins (in CACH) or clear abnormally folded proteins (in AXD). Conversely, prior knowledge about the pathophysiology of three other leukodystrophies, X-ALD, PMD, and MLD, allowed a straightforward design of rational therapeutic approaches. We have thus applied particular molecules to lower the toxic overexpression of a gene (in PMD), anti-oxidant, anti-inflammatory, or neuroprotective compounds (in X-ALD, PMD), or enzyme

replacement therapy (in MLD) to well-characterized mouse models as a prerequisite for considering subsequent clinical trials. All experiments were aimed to identify pharmacological compounds that appear promising with respect to their therapeutic potential in leukodystrophy patients. Indeed, it was possible to initiate the first clinical trials (in X-ALD and MLD).

WP4 - Innovative gene and cell therapies in LDs

Fundamental research has brought new hopes in rare disorders by demonstrating the potential of cell and gene therapy. Partners of this project participated to this effort by developing phase I/II clinical trials based on the combination of gene and cell therapy, using hematopoietic stem cells (HSC) and lentiviral vectors (LV) for X-linked adrenoleukodystrophy (ALD) and metachromatic leukodystrophy (MLD). The objectives of this WP were to intensify this effort in order to:

- pursue and interpret the ongoing clinical trials using HSC and LV for ALD and MLD for further application to select other leukodystrophies (LDs);
- unravel the mechanisms of disease correction in the brain by studying microglia reconstitution after transplantation;
- test the feasibility and therapeutic potential of alternative approaches: a) direct gene delivery to the brain by using intracerebral injection of adeno-associated virus vectors (AAV) or LV for Canavan disease and MLD; b) gene silencing approaches specifically targeting oligodendrocytes (OLs) for Pelizaeus-Merzbacher disease; c) Neural stem cells (NSC) or microglia precursor-based approaches to treat leukodystrophies.

WP5 – Ethical impacts of therapeutic challenges in leukodystrophies

The LeukoTreat project was intended to promote fundamental and clinical research for leukodystrophies. It includes a research database (LeukoDataBase) for patient data sharing at the European level.

WP5 focusd on the perspective of patients and their relatives on the conditions for their participation in the database. WP5 is performed in synergy with the thinking done in WP dedicated to management, where ethical issues were identified and discussed in the LeukoTreat Ethics Committee (LEC). The LEC was set up for the lifetime of the project, and allowed a dialogue between clinicians/researchers in the project and international experts in medical ethics, human sciences and law professionals, together with representatives of patient organizations.

The goal was to gain insight into the perspective of patients/families in parallel to that of health professionals. This should help to identify specific considerations to be addressed in the developing field of registries/databases, where ethical issues differ from those in classical biomedical research. The use of medical data for both care and research blurs the thin line between the two activities, and implies paying particular attention to information to be given to participants in order to ensure transparency and confidence. Two tasks were performed: (i) studying patients'/families' expectations concerning research derived from the LeukoDataBase, data sharing, active participation, and clinical trials, and (ii) helping health professionals in handling the ethical framework of the database and the construction of information

Description of Foreground

WP1 - characterizing leukodystrophies for therapies

Definition of common standard procedures of data registration
Responsible partners: All WP1 partners

In the first 18 months of this project we have harmonized the items related to the collection of clinical data, genetic data and biological samples related to the provided 12 Leukodystrophies (LDs). The items thus chosen have been the background to create the software system for the data base to collect and retrieve clinical and biological samples.

System to collect and retrieve clinical and biological samples
Responsible partners: Soluscience

In parallel, Soluscience started to create the software system to collect a common core of information on all the forms of Leukodystrophies and to work for the specific forms of Leukodystrophies. The software Soluscience system became operational after the decision made by the Leukotreat Ethics Committee in Paris the 1st of July 2011 establishing the criteria for anonymisation of patients anagraphic data sets. This period corresponded to the month 18 of the project. Soluscience has thus developed a generic “common core” service to select patient and data to recover like in a common database system. This service can do all the needed jobs but it can be complicated for a simple user. Soluscience has then created all needed scripts for queries in this case.
All along the second part of the projecct, Soluscience improved the data base so as to answer requests of various clinical data managers and to correct bugs identified by these ones. The data base is now totally operational and can easily be used by managers who can integrate automatically patients data.

Creation of Mutation database for each gene implicated in LD
Responsible partners: UDA

For the collection of the genomic and cDNA sequences as well as the description of the exonic structure the data have been integrated in the UMD software to create 12 UMD DB.
The collection of most of the mutation data base were completed in the second year but also continued until the end of the project and we implemented the number of mutations for all the 12 LDs.

Collection of clinical data, biological samples and mutations for 500 patients
Responsible partners: All WP1 partners

The collection of data has been performed during the step of the project by different centers in three different countries: Italy, France and Germany.

In short:

- Clinical data have been collected by the centers for a total 252 patients (in total 849 different clinical data).
- Biobank sample data of patients have been collected by the centers for a total of 862 patients (in total 2374 samples)
- 872 mutations have been recorded

During the last 24 months of the project, efforts have been made to validate the data registered into the Soluscience system. This one has required improvements of the software

tools, notably in order to make the request tool more efficient. Samples & mutation data from the different Reference Centers were reimported.
In addition, literature references associated to the patients (and their mutation) recorded in the Leukotreat database were listed and imported in the Leukotreat system in order to be included in the UMD databases.
Finally, after reimportation of the first data from the different reference centers and importation of the new data, the correlation between what was expected and what was really in the Soluscience biobank database in terms of samples and mutation numbers was performed and demonstrates that samples' data are now correctly registered.
Since all patients planned to be recorded for mutations and samples data were integrated in the Leukotreat database, all recorded mutations have been exported for each gene using a tool developed by Soluscience and integrated into the corresponding UMD base. The tool extracts the following information from the Leukotreat database: Leukotreat name coded (to which it adds the prefix LKT_), coding mutation, protein mutation, mutation state (homozygous, heterozygous), gender, literature reference. For CACH, Alexander, MLC and PMD disease a family analysis has been performed prior to importation in order to conserve only one affected patient / family to import the corresponding mutation(s). Number of mutations recorded is given below for each gene.

Gene Name	**Disease**	**Inheritance**	**# mutations recorded**
ABCD1	XALD	Xlinked	222
ARSA	MLD	AR	104
ASPA	Canavan	AR	10
eIF2B1	CACH	AR	0
eIF2B2	CACH	AR	19
eIF2B3	CACH	AR	2
eIF2B4	CACH	AR	9
eIF2B5	CACH	AR	91
GALC	GLD/Krabbe	AR	52
GFAP	Alexander	AD	77
MLC1	MLC	AR	21
PLP1	PMD	Xlinked	265
		TOTAL	872

AR autosomic Recessive, AD Autosomic Dominant

Epidemiological analysis: results.
Responsible partners: UKE

A tool for estimating birth incidences of leukodystrophies was proposed by the partner UKE. The concept was discussed and approved for use in the LeukoTreat project at a special meeting in Hamburg on December 7, 2012.

For the calculations performed, the numbers of diagnosed patients have been used as follows. To the numbers of patients whose full clinical data had been transferred to the LeukoTreat database, we added numbers of such patients with confirmed diagnoses where the clinical data were either incomplete or where informed consent to transnational data

transfer was not available. (As those patients are only being counted and have only their birth year recorded, data protection issues did not arise.)

The following national birth figures (for the year 2000) were used as an average reference:

Germany 750.000 births per year
France 800.000 births per year
Italy 540.000 births per year
Total 2.090.000 births per year for the area covered by LeukoTreat.

Using these data, the following estimations of birth incidences in the countries of the LeukoTreat project (France, Germany, Italy) were obtained.

Incidences (per 100 000 life births) in Germany / France / Italy:

Disease	Germany	France	France / Italy
PMD	0.4	0.44	0.34
VWM (CACH)	0.2	0.17	0.16
X-ALD	0.9	0.35	0.26
MLD	0.8	0.65	0.46
Alexander	-	0.27	0.21
Canavan	(0.1)	-	-
MLC	-	-	0.30
Krabbe	0.4	-	0.26

Genotype/phenotype correlation: results
Responsible partners: IGG, UDA

To ensure a uniform codification and classification, the diseases have been defined by OMIM (Online Mendelian Inheritance in Man) number and all mutations have been described according to current mutation nomenclature guidelines (http://www.hgvs.org/mutnomen), ascribing the A of the first ATG translational initiation codon as nucleotide +1.
Data from a total of 746 LD patients with known genotype were available in the LeukoDB classified as Pelizaeus-Merzbacher disease (315 pts), X-linked Adrenoleukodystrophy (222 pts), Metachromatic Leukodystrophy (64 pts), Childhood ataxia with central hypomyelination (89 pts), Alexander (77pts), Globoid leukodystrophy (30pts), Megalencephalic leukodystrophy with subcortical cysts (20), Canavan (6 pts).
Whenever possible a clinical severity score assigned to patients has been used to make genotype-phenotype correlation studies more effective.
Generally, results from genotype-phenotype correlation studies have confirmed the already-known genotype-phenotype correlations for PMD.

However, very interesting results have been obtained when specific gene-mutations were in silico tested for their potentially functional relevance using the UMD (Universal Mutation Database) established by UDA for LDs. Indeed, this tool has allowed us to reliably re-classify some genomic variations (e.g., missense mutations vs splicing mutations). In particular approximately 10% of PLP mutations previously classified as missense mutations were *in silico* predicted to interfere with the splicing process resulting in altered PLP1 RNA transcripts. Whenever possible, the prediction was confirmed on the RNA samples. These results are very important in Pelizaeus-Merzbacher disease since a phenotype-genotype correlation exists and therefore knowledge of the pathogenetic effect of the mutations is fundamental for genetic counselling including prognosis and decision on therapeutic strategies.

Establishment of the diagnosis assistance tool.
Responsible partners: Soluscience

Soluscience has developed new tools to select homogeneous cohort among patient recorded in database mainly by improving the “Query” services. The “Query” services allow user to write complicated query among all objects contained in database. For that user can define:

- Variable list you can use to write different mathematical formula. Each variable can use all other variable previously defined.
- A filtering expression among a table (Patient by example) or an object list (Patient's list by example). It's possible to define many filtering expression to compute many queries in same times.
- By filtering expression user can define many expression to display intels about object checking the filtering expression.

The result of simple query is a matrix list, one by filtering expression. Of course you can publish these matrix in open office spreadsheet. The mathematical formula can use a large library of mathematical function, at this day it's exist more than 2000 mathematical functions you can use on different type of data contained in database:

- Simple data like string, integer, float number, date....
- Matrix of simple data
- Vector of simple data
- Vector of matrix
- Matrix of matrix
- Tree of string.
- Imaging. In this case, database record link to one or many file.

By example, it's pretty easy to record monitoring of patient in a matrix and to use Fourier function to compute different frequency wen can find in a signal.

Unfortunately, the project itself was not very well adapted to test diagnoses tools because:

- We have studied known leukodystrophy and genetic analysis gives immediately the type of leukodystrophy.

- There was no real modeling of each kind of leukodystrophy in order to limit number of parameter we can use to characterize each kind of leukodystrophy. It's only now we have started to do this modeling.

The project was finally mainly used to collect data about lot of patients in Europe which required a strong effort and which then prevented the modeling the different kind of leukodystrophy. In short, the consortium has now tools to perform for diagnoses help but we don't have formula to apply them yet. It will be the purpose of a future project.

Clinical trials on LDs: establishment of the tools for the generation of patients cohorts and electronic clinical forms
Responsible partners: Soluscience

Soluscience has developed many tools to handle clinical trials. All forms developed during the project can be used inside a clinical trial. Platform makes a warning for user when he selects a patient included in a clinical trial. If user fills a form included in a clinical trial for an included patients, the forms will be tagged with id of clinical trial index of session and index of experiment inside sessions.
A clinical trial is a list of three groups:

- A clinical trial is also called a study.
- Inside a study are located one or more session, numbered from 1 to number of sessions
- Inside a session are located experiments (or forms), numbered from 1 to number of experiments in session.

A clinical trial studies subject, not only human, but if it's not humans that are observed, we call that pre-clinical trial. So an experiment is located by 4 data:

1. Patient
2. Study id
3. Index of session in study
4. Index of experiment in session

All forms developed in the system can be natively used like an experiment and platform can tag it.
Additional services were in addition developed by Soluscience to handle clinical trial:

1. Design of session to define list of experiments of a session, id of session model, maximum number of patient, type of clinical trial and configuration of each experiment during the clinical trial. Each form has a configuration from user but if patient is included in clincial trial it's the configuration of clinical trial that is used.
2. Design of study to define list of session in clinical trial, list of possible treatment, possible type of randomization.
3. Two services to create instance of study and session from template designed in previous services. At this level you can define main investigator, number of patient's group, type of protocol, promotor code, id EudraCT, real treatment, randomization, strate....
4. Patient's validation to include them from different criteria. At difference with lot of e-crf including patient is a special session, the first one where patient are studied to know if they can do clinical trials.
5. Many services to follow clinical trial, warning for adverse events, monitoring of captured data, automatic generation of case report form.

We can also notice that the platform provides an Electronic Health Record, and e-CRF and also a LIMS to handle biological sample. It's interesting in the case of rare disease because data captured during a clinical trial can use as clinical data for patient too in the future.

WP2 - Biomarkers for leukodystrophies treatment

Validation of identified biomarkers for LDs

- **NAA-NAAG, marker of OLs functions (M1- M42)**

Responsible partners: UDA, UPD-P7, UCL and CMSUC

In one specific task, we confirmed that high CSF concentrations of the peptide neurotransmitters N-acetylaspartylglutamate (NAAG) are specifically found in leukodystrophies with a primitive defect in myelination such as PMD related to PLP1

mutations and PMLD related to CJC2 mutations in comparison with control and demyelinating leukodystrophies. Further analysis using CSF, urines and serum of “control” patients at different age during brain development are need to use this marker for monitoring therapeutic options at early stage of the disease. The concentrations of the peptide neurotransmitters N-acetylaspartate (NAA) are also raised 1000-fold in Canavan’s disease, a leukodystrophy related to aspartoacylase deficency.

Both NAA and NAAG have been shown to activate metabotropic glutamate receptors and NMDA receptors in neurons, and excessive NMDA receptor activation is well known to cause neuronal death. We have recently demonstrated that NMDA receptors are present both on mature myelinating[1] oligodendrocytes and on oligodendrocyte precursor cells (OPCs)[2]. Furthermore, block of oligodendrocyte NMDA receptors reduces the loss of action potential propagation in myelinated axons that is caused by ischaemia[3], demonstrating the potential for activation of oligodendrocyte NMDA receptors to cause demyelination. These data raise the possibility that an elevated concentration of NAA or NAAG in the leukodystrophies could cause myelin loss by activating oligodendrocyte NMDA receptors. However, as the subunit composition of these receptors differs from that of neuronal receptors[1], it was unknown whether NAA and NAAG will affect the oligodendrocyte NMDA receptors.

In an additional task we tested the effect of NAA and NAAG on oligodendrocytes, and their effect on the oligodendrocyte precursor cells (OPCs), as well as investigating how oligodendrocyte lineage cells can utilize NAA when myelinating axons. Our data rule out the hypothesis that an elevation of NAA or NAAG concentration in leukodystrophies generates an excessive activation of NMDA receptors in oligodendrocyte lineage cells and thus damages the cells[4]. Despite the fact that NAA and NAAG can generate a small rise of calcium concentration in OPCs (detected in ~ 20% OPCs) it does not regulate OPC proliferation, OPC differentiation or myelination.

However, we found that myelinating oligodendroytes express the transporter NaDC3 which may allow them to utilize NAA for myelin synthesis[5]. The transporter is upregulated with differentiation, both during development and remyelination. NAA can increase oligodendrocyte survival during glucose deprivation, but NAA cannot substitute for glucose for myelin synthesis.

In summary, we have identified a transporter that allows oligodendrocyte lineage cells to utilize NAA for myelin synthesis and demonstrated that even at pathological concentrations neither NAA nor NAAG are toxic for oligodendrocyte lineage cells. The high concentrations of NAA and NAAG found in disease may indicate an inability of oligodendrocytes to transport and utilise NAA and NAAG for energy and myelin formation, and thus could be a consequence than a cause of the disease. Therefore, NAAG CSF content could represent a unique marker of oligodendrocytes dysfunctions.

- **eIF2B activity, marker of cellular stress (M1-M36)**

Responsible partners: UDA, UMAN, UPD-P7

The work on using the eiF2B activity as a CACH/VWM biomarker has been very successful. The eIF2 factor is a key regulator of the stress pathway. Its preponderant role in white matter homeostasis has been demonstrated by the defect in its activator, eIF2B (eukaryotic initiation factor 2B), found in the CACH/VWM leukodystrophy. We demonstrated that eIF2B activity can be measured in extracts from patients’ transformed lymphocytes and is a reliable diagnostic and prognostic biomarker for CACH/VWM. Indeed, eIF2B activity is always <77.5% in cells from affected patient carrying eIF2B mutation. This assay requires a source of pure substrate for eIF2B: the eIF2 factor. We have demonstrated that human eIF2 can be expressed and purified from yeast in a functional form, without the need of animal tissues. This recombinant human eIF2 is suitable for performing eIF2B activity assays in patients’ cells and can be used as a diagnostic tool for measuring the impact of eIF2B mutations that cause human disease. Availability of such substrate opens the perspective to measure the eIF2B activity in routine diagnostic and to further investigate the stress pathway in other neurodegenerative disorders[14].

Identification of new biomarkers in LDs

- **Markers of oxidative stress underlying axonal degeneration**

Responsible partners: IDIBELL

Regarding oxidative damage biomarkers for leukodystrophies, we applied redox proteomics to X-ALD, PMD/SPG2 and MLD mouse models. We identified between five to twenty one proteins, depending of the mouse model, related to energy metabolism (glycolysis, citric acid cycle, electron transport chain) as higher oxidized in central nervous system in LDs[6-12]. Further, we studied the role of oxidative damage on the physiopathogenesis of X-ALD. We provide evidence of impaired mitochondrial metabolism in a peroxisomal disease, as fibroblasts in patients with X-ALD cannot survive when forced to rely on mitochondrial energy production, i.e. on incubation in galactose. Oxidative stress induced under galactose conditions leads to mitochondrial damage in the form of mitochondrial inner membrane potential dissipation, ATP drop and necrotic cell death, together with increased levels of oxidative modifications in cyclophilin D protein. Notably, treatment with antioxidants rescues mitochondrial damage markers in fibroblasts from patients with X-ALD, including cyclophilin D oxidative modifications, and reverses cyclophilin D induction in vitro and in vivo. These findings provide mechanistic insight into the beneficial effects of antioxidants in neurodegenerative and non-neurodegenerative cyclophilin D-dependent disorders[10,13].
Next, we sought to investigate the noxious effects of C26:0 on mitochondria function. Our data indicate that in X-ALD patients' fibroblasts, excess of C26:0 generates mtDNA oxidation and specifically impairs oxidative phosphorylation (OXPHOS) triggering mitochondrial ROS production from electron transport chain complexes. This correlates with impaired complex V phosphorylative activity, as visualized by high-resolution respirometry on spinal cord slices of mice. Altogether, our results illustrate some of the mechanistic intricacies by which the excess of a fatty acid targeted to peroxisomes activates a deleterious process of oxidative damage to mitochondria, leading to a multifaceted dysfunction of this organelle. These findings may be of relevance for patient management while unveiling novel therapeutic targets for X-ALD[11].

- **Identification of new lipidic biomarkers in LDs**

Responsible partners: AMC

Metabolomics involves the comprehensive analysis of basically all metabolites produced during metabolism in any organism including human patients and can provide valuable information on any disease state next to genomics and proteomics. In this project we have focused on a subset of metabolomics called lipidomics as a powerful tool to identify new biomarkers being diagnostic or prognostic markers of a disease state. The reason for selecting lipidomics is that the myelin sheath which is such a central element within the group of leukodystrophies (LD) is particularly enriched in lipids, and LD gene defects usually disrupt lipid homeostasis as the primary or secondary consequence of the genetic defect. The achievements within this project are as follows:

1. We have developed a lipidomics pipeline which allows the comprehensive analysis of basically all (phospho)lipid species in different tissues and body fluids. In order to be able to perform this task we have developed automatic compound identification, isotope correction, statistical analysis and finally visualization of the data so that abnormalities can be identified almost by eye.
2. We have used the pipeline described above to identify altered lipids in cells from patients suffering from Pelizeaus Merzbacher disease as well as X-linked adrenoleukodystrophy.
3. We have detected a range of altered lipids in Pelizaeus Merzbacher and X-linked adrenoleukodystrophy mutated mouse brains as well as in spinal cord tissue and plasma samples from ALD and Pelizaeus Merzbacher disease patients.

4. We have also identified a whole series of altered lipids in brains from mice suffering from aryl sulfatase deficiency (a model for metachromatic leukodystrophy).
5. Finally, we have developed a method to measure psychosine and have used this to study this biomarker in brain samples from control and Twicher mice.

Summarizing this part of the work much progress has been made during the project in developing the lipidomics pipeline which holds great promises for the future and has already given us valuable insight into the lipid abnormalities in several of the leukodystrophies including X-linked adrenoleukodystrophy, Pelizaeus Merzbacher disease and metachromatic leukodystrophy.

WP3 – Pharmacological strategies to treat leukodystrophies

Cholesterol-derivatives for remyelination and neuroprotection in LD
Responsible partners: Trophos, UDA

Partner TROPHOS has identified and developed a 'first-in-class' series of cholesterol derivative compounds with a survival benefit on both neuronal and non-neuronal cells, currently in a phase II/III clinical trial for spinal muscular atrophies. In collaboration with partner UDA, our objective was to establish the potential therapeutic effect of the cholesterol derivative olesoxime (TRO19622), selected for its additional promyelinating properties, and to test its efficacy to prevent or repair the myelination in the PLP^{overexpressor} mouse model of Pelizaeus-Merzbacher Disease (PMD) (PLOA), or the axonopathy in the PLP^{null} mouse model of type-2 spastic paraplegia (SPG2).

We first evaluated the efficacy of TRO19622 on survival, weight, behavior, and neuropathological defects in PLP^{overexpressor} mice. We were unfortunately unable to see any effect on survival, weight and behavior. Neuropathological analysis revealed a beneficial effect of TRO19622 on the number of oligodendrocyte precursors in the corpus callosum, but not on astrogliosis or microgliosis. While TRO19622 has improved the number of immature oligodendrocytes the treatment did not promote the remyelination and motor recovery in this model, different from previous observations in chronic but transitory demyelinating states induced by cuprizone or LPC.

In a second approach, we initiated the evaluation of TRO19622 in PLP^{null} mice and assessed the efficacy of treatment begun in 3-month-old mice, before the onset of behavioral symptoms. We showed a delay of 3 months in the appearance of some behavioral symptoms as well as in the slowing of nerve conduction velocity observed with auditory evoked potentials, while other parameters altered (body weight, grip strength, working memory) were not improved by the treatment. Until now, neuropathological analysis did not identify cellular substrates responsible for the beneficial effects observed with TRO19622 treatment. However, data are still under evaluation and an effect of olesoxime on axonal degeneration or oxidative stress is not yet ruled out. Further evaluation is thus required to fully understand the therapeutic effect of TRO19622 on anxiety-related dysfunction and the slowing of nerve conduction observed in PLP^{null} mice. Nevertheless, the possibility of delaying 3 months the appearance of certain symptoms in PLP^{null} mice constitutes a hope for improving the quality of life in SPG2 patients.

Anti-oxidative and anti-inflammatory dietary supplement in LD
Responsible partners: MPG and IDIBELL

MPG have evaluated the therapeutic effect of applying the dietary supplement Turmeric to PLOB mice as well as to *Plp1*^{rumpshaker} mice, which represent a genuine model of PMD caused by a point mutation of the *PLP1* gene. *Plp1*^{rumpshaker} mice are considerably more severely affected than PLOB mice. Among different application methods (injection, oral) the application of Turmeric as a dietary supplement to the chow was found efficient and less stressful to the animals. Indeed it was possible to improve the pathology of *Plp1*^{overexpressor} and *Plp1*^{rumpshaker} mice. Importantly, mortality of *Plp1*^{rumpshaker} mice was delayed in the

experimental group, in agreement with the amelioration of various neuropathological markers. Upon treatment of *Plp1*^{overexpressor}, a clinical score was evaluated, and the outcome for motor capabilities was additionally measured by a limb-sliding assay. We have focused on the cerebrospinal tract of the spinal cord, as demyelination of these long-projecting axons is likely the cause of deteriorating motor capabilities. Indeed, neuropathology and the number of myelinated axons were improved in the experimental group. As expected, the mechanism of action does not involve a reduction of Plp1-mRNA abundance. There is also no indication that the most likely active ingredient of Turmeric (Curcumin) would affect the intracellular trafficking of proteolipid protein (PLP), which may reflect that Turmeric is likely beneficial in the PMD mouse model due to its anti-inflammatory and anti-oxidative capacity. As a next step towards a future clinical trial, we aim at the pre-clinical application of a Curcumin preparation with improved bioavailability in combination with an altered lipid diet.

Whereas, the aim of IDIBELL was to find novel pharmacological treatment options for X-ALD. A first objective was to investigate the effect of FDA-approved antioxidant compounds, such as lipoic acid, N-acetylcysteine, coenzyme Q, creatin, and vitamin E, alone or in combination, in a preclinical setting using a mouse model of X-ALD. The combination of creatin and coenzyme Q was ineffective, however the treatment with a combination of lipoic acid, N-acetylcysteine and vitamin reversed: i) oxidative stress and lesions to proteins, ii) immunohistological signs of axonal degeneration such as axonal spheroids, and iii) locomotor impairment.
These results provide conceptual proof of oxidative stress as a major causative disease-driving factor in X-ALD, thus warranting translation into clinical trials for X-AMN patients and inviting assessment of antioxidant strategies in other diseases with axonal degeneration in which oxidative damage may play a role. We thus launched a phase II clinical trial in October 2011 with 13 AMN patients (EUDRACT 2010-024084-40; NCT01495260), which was completed in September 2013. The trial has validated 5 different oxidative lesion biomarkers of use to assess biological activity of antioxidant compounds in future clinical studies. The analysis of potential clinical benefits using clinical scales for spasticity, walking tests and MRIs, is on progress. Positive results would encourage randomized versus placebo, multicentric, international trials with the combination of antioxidants. The information is being collected in LeukoDB, of great value for natural history of disease and for facilitating the inclusion of these patients in future trials.

Development of strategies to achieve the release of misfolded proteins

- **Pharmacological release of GFAP in AXD**

Responsible partners: INSERM

Alexander disease (AXD) is a leukodystrophy caused by dominant mutations in the *GFAP*-gene encoding Glial Fibrillary Acidic Protein, the main intermediate filament protein in mature astrocytes. This degenerative disease is characterized by the presence of Rosenthal fibres, large aggregates of mutant GFAP associated with small heat shock proteins, in dystrophic astrocytes, and loss of myelin. The objective of partner INSERM was to assess potential therapeutic strategies in two models of Alexander disease, two lines of GFAP^{knockin} mice (one with mutation in the tail-domain and one with mutation in the rod-domain of GFAP).

In vivo, both mouse models present with dystrophic astrocytes with aggregates similar to Rosenthal fibres. In astrocyte cultures, one line expressed 20% aggregates, making it appropriate for testing selected compounds inducing chaperones and autophagy. In a pilot compound application, we observed a considerable elimination (or prevention of formation) of aggregates in mutant astrocytes. We have selected two drugs without toxic effect, the one stimulating autophagy and the other one known for its neuroprotective effect and its role in the proteasome regulation, to pursue the study. *In vitro*, both drugs and their association decrease the number of aggregate-bearing astrocytes (decrease of aggregates from 26-56%

and of aggresome-like structure from 40-75%). We are now ready to test the effect of these drugs in our two lines of GFAP^{knockin} mice.

- **Small compound screen to rescue misfolded ABCD1 in ALD**

Responsible partners: AMC

X-ALD is one of the most puzzling inborn errors of metabolism of the CNS. All X-ALD patients have mutations in the *ABCD1* gene and accumulate very-long-chain fatty acids (VLCFA). The clinical spectrum, however, ranges from a neurodegenerative disease of the spinal cord in adult males and females (adrenomyeloneuropathy, AMN) to a fatal brain disease in boys (cerebral-ALD). Treatment options for X-ALD are very limited. For some young boys who develop cerebral-ALD, a bone-marrow transplant may be curative. Unfortunately, this can only be offered during a narrow therapeutic window, which is often missed. For patients with the AMN phenotype, which represents 85% of all patients, no curative or disease-modifying therapy is available. A lipid preparation termed 'Lorenzo's oil', also known from a movie of the same name, does reduce plasma levels of VLCFA, but available evidence suggests it does not halt disease progression in patients. There is a dire need for new therapeutic options.
By using cultured skin fibroblasts from X-ALD patients, partner AMC has identified bezafibrate as a VLCFA-lowering drug. Our experiments demonstrated that bezafibrate inhibited the enzyme ELOVL1, thereby reducing the synthesis and levels of VLCFA. The results of these studies in patient cells had been reported at scientific and patient meetings and we were aware of patients starting with bezafibrate medication by themselves. We initiated a clinical trial with bezafibrate to "evaluate the effect of bezafibrate on VLCFA metabolism in men with X-ALD". Our goal was to test whether bezafibrate has therapeutic potential *in vivo*. The aim was to initiate, complete and report the results of the clinical trial in the shortest time possible. Ten patients with the AMN phenotype enrolled in the trial. The endpoint was the effect of bezafibrate treatment on VLCFA levels in plasma, but more importantly blood cells. Unfortunately, bezafibrate treatment did not lower VLCFA in blood cells of X-ALD patients. This is most likely due to limited bezafibrate levels that could be reached in plasma (25 µmol/L) while in cells bezafibrate was active at >200 µmol/L. Bezafibrate thus appears to have no therapeutic utility in X-ALD. Our future work is aimed at indentifying highly specific ELOVL1-inhibitors that act at much lower concentrations than bezafibrate and are well tolerated in patients.

Small compound screen to enhance protein translation in LD

Responsible partners: UMAN

The identification of the *EIF2B* gene (encoding the eukaryotic translation initiation factor eIF-2b) as causative of Childhood Ataxia with Central Nervous System Hypomyelination (CACH) has emphasized the relevance of regulated protein synthesis ('translation') in the white matter of the brain to achieve rapid responses to cellular stress. The observed abnormally elevated stress response is believed to contribute to disease pathology. eIF2B is central to the normal control of the stress response that is also modulated by environmental factors. *EIF2B* mutations reduce its activity but do not eliminate it. In many cases activity remains above 50%. Molecules able to interact and boost the basal eIF2B activity could represent lead compounds that could be developed into therapeutic strategies to prevent the myelin breakdown and neuronal degradation associated with disease. In order to identify potential therapeutic molecules that may correct the consequences of EIF2B related diseases, partner UMAN has thus established a medium-throughput compound screening system that was applied to a yeast model of eIF2B mutations.

Libraries of small compounds already in clinical use for other conditions or with 'drug-like' qualities, were screened in a yeast cell-based system using an eIF2B-dependent reporter assay. 'Hit compounds' were re-screened against multiple EIF2B-mutant strains in the

primary assay and the best candidates were screened in a range of follow-up biochemical assays to assess their general impact on eIF2B-related functions. The most promising candidates were protease inhibitors. However they did not act directly on eIF2B itself, as eIF2B protein levels remained unaltered. Thus none of the compounds assessed appeared suitable for taking forward for screening in more complex systems such as patient-derived cells.

Development of strategies to modulate gene expression in LDs

- **Pharmacological induction of ABCD2 in ALD**

Responsible partners: MUW

The genetic basis of X-linked adrenoleukodystrophy (X-ALD) are mutations in the peroxisomal fatty acid transporter ABCD1. Previously, partner MUW and others have demonstrated that the overexpression of a closely related transporter, termed ABCD2, can functionally compensate for the loss of ABCD1. Different expression patterns of ABCD1 and ABCD2 in disease-relevant cell types were suggested as the reason why the endogenous ABCD2 cannot compensate for ABCD1 deficiency in X-ALD. This part of the project thus focused on the preclinical evaluation of a new therapeutic strategy aiming at a pharmacological induction of ABCD2 gene expression in order to compensate for the functional loss of ABCD1 in X-ALD. Currently, the only curative therapy is allogeneic hematopoietic cell transplantation. We thus systematically explored major immune cell types derived from CD34-immunopositive stem cells for the expression pattern of ABCD1 and ABCD2, and the metabolic impairment in blood-derived immune cells from X-ALD patients and controls. By this approach, we identified monocytes as the most severely impaired blood cell type in X-ALD.
These findings contribute to our understanding of why hematopoietic stem cell transplantation can halt the brain inflammation in X-ALD. In the next step, we elucidated the level of induction of ABCD2 expression in macrophages that would be expected to mediate therapeutic benefit. This knowledge is of crucial importance for further evaluation of drug effects and estimation of potential efficacy of compounds for X-ALD treatment. Furthermore, we have investigated a variety of compounds for their capacity to induce ABCD2 *in vitro* in monocytes. The best candidate from this screening was Isotretinoin, a retinoid in clinical use for treatment of severe acne. The effect of Isotretinoin on ABCD2 expression was analyzed in monocytes of acne patients at regular blood controls, before and during oral treatment with Isotretionoin. However, in this study Isotretinoin failed to promote a substantial induction of ABCD2. Additional compounds, or possibly combinatorial treatments, have to be considered in order to achieve the required activation of ABCD2 expression in monocytes.

- **Pharmacological regulation of PLP-gene expression in PMD**

Responsible partners: MPG

The majority of PMD cases are caused by the duplication of a genomic segment that includes the *PLP1*-gene, which encodes the most abundant constituent of central nervous system myelin, proteolipid protein (PLP). The duplication leads to the overexpression of the gene, with dramatic pathophysiological consequences. Partner MPG has performed a therapy trial in the relevant *Plp1*^{overexpressor} mouse model of PMD (PLOB) aimed at lowering Plp1 overexpression by applying a progesterone antagonist (Lonaprisan). The therapeutic concept is that progesterone antagonists would lessen the abundance of Plp1 mRNA and thereby lower the toxically high dose of protein. The Plp1 mRNA expression level in PLOB mice was increased 1.8-fold compared to controls and reduced by 15% upon Lonaprisan treatment. The motor capabilities, as measured by a limb-sliding assay, were significantly improved after Lonaprisan application. Quantification based on transmission electron microscopy revealed an increase in the number of myelinated axons after Lonaprisan treatment by 30%. Lonaprisan treatment also reduced microgliosis, astrogliosis, lymphocyte

infiltration and oligodendrocyte death according to quantitative PCR and immunohistochemistry using neuropathological markers. Interestingly, Plp1 mRNA overexpression correlated to the axon number and the phenotype. Partner MPG suggests that these data provide proof-of-principle that pharmacologically lowering the overexpression of Plp1 may indeed constitute a rational therapy for PMD patients harbouring Plp1 gene duplications.

Delivery of drugs to the CNS
Responsible partner: UKB

The main issue for enzyme replacement therapy (ERT) in metachromatic leukodystrophy (MLD) is the blood-brain barrier (BBB), which limits the delivery of arylsulfatase A (ARSA) from the blood to the brain parenchyma. Using arylsulfatase A (ARSA) deficient mice as a model of MLD, partner UKB pursued two strategies to overcome the BBB.
In a first approach, we tested implantable minipumps delivering ARSA directly into the cerebrospinal fluid of one brain hemisphere, an approach called intracerebroventricular ERT. This study revealed that ARSA infused for four weeks efficiently enters the brain and spreads over large distances. Lysosomal storage of all storing cell types was completely reversed in the infused hemisphere. Sulfatide clearance declined with increasing distance from the infusion site. The extent of sulfatide clearance did not only depend on local ARSA concentrations, but also on the cell type. Microglia were most responsive to treatment as microglial storage disappeared also distant from the infusion site. Oligodendroglial storage, on the contrary, was cleared in the infused hemisphere but retained in the other hemisphere, indicating the requirement of high ARSA concentrations for correction. Most importantly, treatment corrected the ataxic gait of ARSA-deficient mice indicating reversal of CNS dysfunction. The profound histological and functional improvements, the requirement of low enzyme doses and the absence of immunological side effects suggest intracerebroventricular ERT as a promising treatment option for MLD. The success of our preclinical studies contributed to the implementation of a phase I/II clinical trial sponsored by Shire Human Genetic Therapies, Inc., Lexington, USA. MLD patients below 9 years of age receive recombinant ARSA (10, 30 or 100 mg) via intrathecal drug delivery every other week for a total of 38 weeks (20 injections). In this set-up, ARSA is infused into the cerebrospinal fluid of the spinal cord. Outcome measures are safety and gross motor function.
In a second approach we modified the ARSA enzyme to improve its ability to overcome the BBB when applied via the conventional intravenous route. Therefore we generated chimeric (or fusion) proteins in which ARSA was linked to peptides, which have been previously shown to cross the BBB. The most effective fusion protein was selected by different cell culture tests and mouse studies. It is termed ARSA-ApoE-II and composed of ARSA and a peptide derived from the human protein apolipoprotein E, an abundant plasma protein involved in lipid metabolism. Following intravenous injection, 54% more ARSA-ApoE-II reached the brain of ARSA-deficient mice compared to unmodified ARSA. To determine the therapeutic potential of ARSA-ApoE-II, ARSA-deficient mice were treated by four intravenous injections in weekly intervals. Treatment with unmodified ARSA reduced sulfatide storage both in kidney and in brain by 22%, whereas ARSA-ApoE-II diminished sulfatide storage by 37% and 38%, respectively. Thus, ARSA-ApoE-II cleared 1.7-fold more sulfatide from the tissue than wildtype ARSA. Minor variations in residual ARSA enzyme activity between 0 and 5% of normal determine the entire spectrum of clinical manifestations of MLD from early-onset forms to healthy individuals. It is thus likely that the observed increase in brain delivery and sulfatide clearance may significantly improve the therapeutic benefit of ERT. Future long-term studies in ARSA-deficient mice and MLD patients are required to test this notion.

WP4 - Innovative gene and cell therapies in LDs

HSC gene therapy using lentiviral vectors for “therapeutic” protein delivery

- **Evaluation of the efficacy and safety of HSC gene therapy using LV**

Responsible partners: INSERM, HSR

- ALD: 4 ALD patients have been treated by HSC gene therapy using LV with a follow-up of 6y, 6y, 4y1/2 and 2y1/2 years. The results demonstrated that LVs are able to transduce multi-potent long-term repopulating hematopoietic cells. The ALD protein was expressed in 8-11.5% of myeloid and lymphoid cells, 2½-6 years after gene therapy. In respect to clinical efficacy, the long-term follow-up of the 4 patients confirms that HSC gene therapy with LV has been able to arrest the progression of cerebral demyelinating lesions of X-ALD, as observed after allogeneic HSC transplantation (HCT). In term of safety, all tests searching for replication competent LV (RCL) are negative in the 4 treated ALD patients up their last follow-up. Concerning potential genotoxicity, there is no evidence of dominant clone due to insertional mutagenesis, of higher retrieval of LV insertions in oncogenes than in other genes. This follow-up demonstrates, up to now, the safety of HSC gene therapy with LV in X-ALD patients.

- MLD: A total of 7 pre-symptomatic late-infantile (LI) MLD patients were treated; post-treatment follow-up ranges in between 6 and 36 months. Pre-symptomatic LI MLD patients were treated from 2 to 11 months before the expected disease onset, determined according to the reported age at onset of the disease in the affected older siblings. The clinical efficacy of such therapeutic strategy to arrest or even reverse cerebral demyelination and its safety in respect to insertional mutagenesis was evaluated for the first three LI MLD patients in which the post-treatment follow-up is ≥ 2 years. The results demonstrate that LVs are able to transduce multi-potent long-term repopulating hematopoietic cells. Between 45 and 80% of HSC were corrected. None of the 3 treated patients developed the severe course of disease reported in their older brother or sister or observed in untreated late infantile MLD patients. Basically, cerebral demyelinating lesions seen at brain MRI in untreated subjects were prevented and treated patients had essentially normal or near normal motor and cognitive functions up to the last follow-up. The follow-up of the treated patients is continued. In terms of safety, the results were the same as in the ALD trial: no evidence of dominant clone due to insetional mutagenesis, of higher retrieval of LV insertions in oncogenes than in other genes.

- **Regulation of therapeutic gene expression in HSCs for safe and efficacious HSC gene therapy**

Responsible partners: INSERM, HSR

Overexpression of galactocerebrosidase (GALC) enzyme (which is deficient in Krabbe leukodystrophy) in HSCs is toxic for these cells. miRNA-regulated LV for GALC gene transfer were therefore explored: the use of a LV containing the target sequence of an miRNA exclusively expressed in hematopoietic cell progenitors (miR126) allowed safe transduction of HSCs and GALC expression only in their differentiated progeny, including myeloid cells. The efficacy of the miR-126 based regulatory system was also compared to a transcriptional strategy based on use of the myeloid-specific CD11b promoter to target GALC expression to the differentiated HSC progeny. The GALC.126T LV effectively protected human and murine HSC from GALC toxicity and showed better expression of GALC in progeny than the use of lenti-CD11b-GALC. In Trs mice, a mouse model of Krabbe leukodystrophy, HSC gene therapy using the miR126-regulated LV was substantially more effective than wild type HCT to ameliorate the disease phenotype. These findings are currently being reproduced in the most severe disease model (Twitcher mouse) of Krabbe leukodystrophy. These pre-clinical experiments provide the proof-of-principle of a novel gene therapy approach that could potentially be implemented in clinics.

Development of new sources of brain microglia

Responsible partners: HSR, INSERM, UKB

We demonstrated that a short-term wave of brain infiltration by a fraction of the transplanted early hematopoietic progenitors occurs upon HCT, independently from the administration of a preparatory regimen and from the presence of a disease state in the brain. Importantly, the use of a conditioning regimen capable of exerting an ablative effect on functionally-defined CNS-resident myeloid precursor
s favors turnover of microglia with the donor cells, very likely mediated by local proliferation of early immigrants. The administration of a myeloablative busulfan dose (and to less extent of irradiation) prior to HCT is associated with the appearance of abundant donor-derived cells with the antigenic features and the morphology of intra-parenchymal microglia. These results have obvious implications for HSC gene therapy as well as allogeneic HCT for leukodystrophies. In particular in the setting of reduced intensity conditioning.
For lysosomal leukodystrophies, it is essential that non-corrected brain cells can recapture with efficacy the enzyme from normal (in the case of HCT) or corrected (in the case of gene therapy) cells. Microglial cells are the source of active lysosomal enzyme in the brain after allogeneic HCT or HSC gene therapy. In vitro studies showed that astrocytes and microglia, but not oligodendrocytes capture well ARSA, the enzyme that is deficient in MLD. Microglia were shown to internalize ARSA via multiple receptors, the mannose 6-phosphate (M6P)-receptor playing a minor role. Importantly also ARSA secreted by microglia cannot be taken up by other cells in a M6P-dependent manner. Therapies of MLD based on substitution of active ARSA (allogeneic HCT, enzyme replacement, HSC gene therapy with LV) seems therefore to be complicated by the low M6P-dependent endocytic rate of ARSA by oligodendrocytes.
In X-ALD, the ALD protein cannot be secreted by corrected microglia, as it is the case for ARSA in MLD and GALC in Krabbe leukodystrophy. There is in addition no evidence of cross-correction of VLCFA accumulation in astrocytes and oligodendrocytes after allogeneic HCT in ALD mice. This raises the possibility that ALD microglia is deficient and that allogeneic HCT or HSC gene therapy with LV corrects only a pool of deficient microglia. We showed that Abcd1$^{-/-}$ microglia differ from wild-type microglia in various parameters in *in vitro* primary cell culture. Abcd1$^{-/-}$ microglia secrete significantly more Il-6 and NO after activation by LPS and had a higher proliferative rate in a non-activated state
Adrenomyeloneuropathy (AMN) is the adult form of ALD. It is characterized by severe spastic paraplegia in adulthood. The pathology involves only the spinal cord and is of axonal degeneration type, not of demyelinating type, as cerebral ALD. The ALD knock-out mice develop an “AMN-like” phenotype without cerebral demyelination. We showed that allogeneic HCT in ALD mice prevents the onset of motor disability and correct many oxidative stress abnormalities that are the hallmark of AMN. The correction of neuropathological abnormalities was only partial. This has implication for the long-term outcome of ALD boys treated with allogeneic HCT or HSC gene therapy for their cerebral disease, but also for gene therapy strategy of AMN.

We aslo attempt to develop new source of brain microglia. These attempts, either starting from HSC or embryonic stem cells failed up to now unfortunately.

Development of new therapeutic strategies using NSCs
Responsible partners: INSERM, HSR

This strategy was assessed in mouse models of MLD and Krabbe disease. Murine (m) neural stem cells (NSCs) transplanted in the cerebral lateral ventricles of Twitcher mice (a murine model of Krabbe disease) engrafted rapidly and long-term (yield of engraftment 0.2% - 3%), producing a functional GALC. Levels of enzyme activity in brain and spinal cord tissues reached up to 50% the WT levels and were enhanced when GALC-overexpressing NSCs were used. Treated mice showed improved walking ability (gait analysis), delayed onset of symptoms (5-7 days) and moderate increase in lifespan (+7 days). These promising therapeutic benefits were reproduced using hNSCs, which showed similar pattern of cell

engraftment, distribution and metabolic reconstitution in both Twitcher and immunodeficient MLD mice. Next, we tested the efficacy of a combined treatment (NSC gene therapy + HSC transplant) performed in neonatal GLD mice to achieve fast and therapeutically relevant GALC activity in the brain. Importantly, NSC gene therapy provided short-term GALC supply in CNS tissues before engraftment of LV-corrected HSCs. Despite no apparent synergy of treatments in terms of GALC activity, combination therapy enhanced benefits when compared to single treatments in terms of: i) reduced storage and astrogliosis: ii) median survival: 195 days using combined strategy (n=6) instead of 70 days for HSC transplant alone (n=9), 45 days for NSC gene therapy alone and 40 days for untreated Twitcher mice (n= 177). The proposed combination therapy might represent a suitable approach for Krabbe disease.
Transplantation of human NSC was also assessed in a mouse model of Pelizaeus-Merzbacher disease (PMD): the PLOA mice that overexpress several copies of PLP genes as most PMD patients. For this purpose and avoid graft rejection, immune deficient RAG2-PLOA mice were generated. Analysis indicate that: i) the majority of transplanted human NSC injected in the rostral forebrain survive and target the corpus callosum; ii) while some of the transplanted cells remain immature, others differentiate in mature oligodendrocytes; iii) finally transplanted cells exert a neuroprotective action through the decrease of microglia and astrocyte activation. Impact of human NSC transplantation on motor phenotype of PLOA mice is currently addressed and should indicate whether these neuroptrotective and pro-myelinating effects are of clinical benefit in this model.

Development of strategies allowing direct delivery of the therapeutic gene to the brain to treat LDs
Responsible partners: UDA, INSERM, UNSW

For MLD, proof-of concept has been made in MLD mice that the intracerebral delivery of AAVrh10 vector expressing the ARSA enzyme is more efficient that the intracerebral delivery of AAV2-ARSA vector to correct rapidly the sulfatide load in oligodendrocytes. Towards a clinical trial, this strategy has been evaluated in normal non-human primates (NHPs). A new method of intracerebral delivery of viral vector has been designed, tested and validated in terms of efficacy and safety. Using this new method of vector delivery, intracerebral injection of AAVrh10-ARSA vector in the brain of NHPs showed expression of ARSA in a > 50% of brain and importantly the expression of ARSA in oligodendrocytes. Toxicological and biodistribution studies were performed in NHPs at 5-fold (in term of vg/cm3 of brain) the vector dose scheduled to be administered to MLD patients. These results in term of efficacy and safety allowed the CTA approval of intracerebral gene therapy for MLD by the french agency for drug administration (ANSM). The trial is now open.
For Canavan disease, an AAV vector expressing the ASPA enzyme (which is deficient in Canavan disease) under the control of specific oligodendrocyte promoter was used. For this purpose, an ASPA-Ko mice was developed and fully characterized. More recently, a mouse model was developed in which ASPA is selectively inactivated in oligodendrocytes and Schwann cells. This conditional mouse line is completely devoid of ASPA in the brain suggesting that oligodendrocytes are the sole ASPA expressing cells in the CNS. Surprinsingly, the use of an AAV vector with an oligodendrocyte specific promoter led to transient expression of ASPA in ASPA-KO mice and mostly in neurons. Yet, this transient CNS expression of ASPA was sufficient to improve neurological functions, histopathology, neurochemical abnormalities, and demyelination. Various modifications of vector and experiments are ongoing to understand why the use of AAV vector expressing the GFP under an oligodendrocyte promoter allows the expression of this reporter protein in oligodendrocytes, and why it is not the case when ASPA is used as transgene instead of GFP.
For Krabbe disease, we also developed an efficient protocol of LV-mediated intracerebral gene delivery in adult and neonatal Krabbe and MLD mice, showing widespread transgene distribution with a single unilateral injection in white matter tracts. Robust enzymatic reconstitution in CNS tissues correlated with amelioration of pathology and, in neonatally

injected Krabbe mice, in delayed onset of symptoms and improved survival. Preliminary evidence suggests that vector and transgene biodistribution and expression could be enhanced using multiple injections (up to three). Based on these results we set up a biodistribution study using LV.hARSA in normal NHPs, with the aim of assessing vector and transgene distribution in CNS and non-CNS tissues following two unilateral injections in the external capsule and thalamus, respectively (studies not funded by the Leukotreat project).
PLP1 duplications are the most frequent mutation found in PMD (60%) and all include the entire PLP genomic region. Therefore, silencing the PLP gene would be a valuable strategy. Towards this aim, we tested a morpholino antisense oligonucleotide strategy. In vitro, morpholinos were demonstrated to silence GFP protein in oligodendrocytes engineered to express the GFP under the control of PLP promoter. We then moved to use vivo-morpholino developed by Gene-tool l® to target the PLP gene. It turned out that these vivo-morpholino have toxicity for oligodendrocytes primary culture. At lower (and less toxic) dose, they failed to silence the PLP gene. This markedly delayed our program. We then designed classical morpholino to silence the PLP gene and tested their efficacy in vitro (oligodendrocytes). However, evaluation of multi-injection of classical morpholino in PLOA mice failed to show robust silencing of the PLP gene. We are now developing a strategy in which in vivo silencing of PLP gene in PLOA mice will be evaluated after intracerebral injection of shRNA targeting the PLP gene and vectorisation with an AAV vector allowing the expression of this shRNA in oligodendrocytes.

WP5 – Ethical impacts of therapeutic challenges in leukodystrophies

Responsible partners: LEM

Studying patients and families' expectations concerning research derived from the LeukoDataBase (LuekoDB)

The work in WP5 consisted in the production of a survey addressing:

- Issues linked to the sharing of personal data, desire to participate and conditions for sharing,
- Utility of such a database for future clinical trials,
- Utility of entering own data in the database.

The survey was addressed to patients, their families (parents and other relatives), or their legal representatives. Due to the characteristics of leukodystrophies, which may affect young children and cause mental impairment, the questionnaire was issued to patients and their families. It was designed with the help of the LEC.

Survey production

A questionnaire was constructed based on a first survey delivered to the French families participating in the annual Families/Scientists meeting of the European Leukodystrophies Association (ELA) in April 2011. In all, 170 printed questionnaires were distributed, together with an accompanying letter explaining the goal of the study, and a stamped envelope for return. The survey was also introduced in an oral presentation during the meeting. Fifty-five questionnaires were returned by the end of September 2011 (response rate 32%) and analysed. This feedback was used to construct the final version of the questionnaire, composed of 36 closed or ranking questions organized in five sections as follows: (i) Respondent's profile (Q1 to Q5), (ii) Point of view on personal health data sharing (Q6 to Q17), (iii) Point of view on family genetic data (Q18 to Q21), (iv) Point of view on respondent's participation in the database (Q22 to Q27), and (v) From the database to therapeutic clinical trials (Q28 to Q36). Space for open comments was provided for a number of the questions. The questionnaire was sent to the LEC for comments and validation. It was then made available in French, English, German, Spanish and Italian. An online version in

each language was produced with the help of the partner Soluscience on Adobe Acrobat X Pro software. The return and analysis of the survey were completely anonymous.

Survey diffusion

The survey was circulated in several ways:

- Via the European network of Leukodystrophies Association ELA. Contact persons were met during the ELA Families / Scientists Meeting in April 2012 to explain the objectives of the research. A number of printed questionnaires, estimated by the contacts, were sent for circulation by them. Patients/families from the French ELA association were asked to answer the revised questionnaire.
- Directly to the patients in the reference clinical Centres in France, Italy and Germany. Resource professionals were identified in the Centres, and printed questionnaires were sent to them. The questionnaire was given to patients/families on the occasion of a medical consultation or hospitalization.
- Online questionnaires were made available on the LeukoTreat website; the links were sent to the contact persons and given on the ELA website.

Survey analysis and results

The responses were entered into an Excel document, and a chi2 analysis was performed for statistical analysis. A qualitative analysis of the comments was performed.
A total of 195 questionnaires were returned, 46 from patients and 149 from relatives (139 parents/mothers and 10 close relatives). Despite a significant difference in the number of answers in each group, the choice was made to analyse them separately. By contrast, an analysis by country proved impossible because of the wide-ranging numbers of answers (130 French (23 patients) /24 Italian (2 patients)/9 Belgian (2 patients)/6 Spanish (1 patient)/26 German (18 patients).
The results of the survey revealed an extremely high motivation for participation in research. As there is no cure for leukodystrophies, there was a clear hope for progress from research. Collecting and sharing patients' data was perceived by the respondents as an important way to help advancement in research and to promote a better understanding of the disease. It was also perceived as a way to obtain better access to clinical trials if developed. Patients/families expressed their motivation to face up to the disease, and get involved in research. Most of them were ready to enter some data by themselves in the database. On the other hand, the survey identified a lack of awareness of the respondents on a number of points that should be taken into account in the construction of the information. A number of respondents were unable to state whether or not they were participating in research that collected personal, clinical and biological data for leukodystrophies. As the database is partly built from clinical data collected from patients as they present for care, patients/families may be unaware that their medical data is being shared for research purposes, even though they signed a consent form for this purpose. Patients' conceptions of biomedical research usually revolve around technical and drug trials involving interventions on human beings. Particular attention should thus be given to helping patients make the distinction between care and research.
A publication presenting the result of the survey is in preparation. Results will be made accessible after acceptance of publication.

2) Helping health professionals in handling the ethical framework of the database and the construction of information

The work performed in WP5 was interlinked with the ethical management performed in WP7 aiming at the promotion of harmonized ethical practices in the Consortium. The work was performed by the ethics research group together with the LEC, the LeukoTreat ethics committee set up for and dedicated to the project.

Production of a charter governing the database

The “LeukoDB Charter” for “Leukodystrophies DataBase Charter” develops the main principles on data privacy, regulation of potential value, and the exploitation of data, as well as issues tied linked to consent, information and return of results. The Charter aims at to ensureing transparency of the LeukodDataBbase functioning operation for the Members and Partners of the LeukoDB Network, informing participants about the data used through the LeukoDB, and informing Members and Partners of the LeukoDB Network about their commitment regarding the LeukoDB functioning operation. The Charter is based upon international reference documents on personal data protection, and right of persons regarding research (international treaties and European directive). It can serve as a model document for other collaborative projects that aim to create a patient database.

Harmonization of information and consent procedures

Models for patient information and consent forms were produced after evaluation of existing documents, and recommendations made by the LEC.
Two harmonized patient information forms were created: one that provides details on the nature, significance, implications and risks of taking part in a database, and one that specifically deals with the use of already existing biological samples within the database. Three versions were developed for each form: one dedicated to the legal representatives of underage patients, another to legal guardians of protected major patients and one for major patients. One information form for patients reaching the legal age of majority was also prepared.
The **Patient Consent Form** is used to confirm that the patient agrees to participate in the database. As with the information forms, three harmonized versions were prepared: one for the legal representatives of underage patients, another for legal guardians of protected major patients and finally one for major patients.
A **decision tree** was drawn to help the clinicians choose the right form according to the specific situation: minor patient, protected major patient, major patient, or patient reaching the age of majority.

Impact and dissemination activities

Regarding the Leuko database (LeukoDB), The ELA foundation (www.ela-asso.com) will be in charge of managing and developing it asof the end of the project.
The LeukoDataBase will be used first of all as a European register of patients. It will not only facilitate recruitment of patients but, knowing the high number of a specific form of leukodystrophy, it will have implications on the design and power of a given therapeutic clinical trial.
The clinical database will facilitate epidemiological data about the overall number of this group of rare disorders. It will also allow to know more about the distribution of each specific form of leukodystrophy in Europe. The Biological Sample database will offer a repository of biological samples to all the research community in order to accelerate and facilitate functional studies on the different forms of leukodystrophies. Finally, the Mutation Data for each gene known to be implicated in leukodystrophies will give the possibility to integrate the clinical epidemiological data and define the hot spot mutations for each form of leukodystrophy so that it will be easier to implement the diagnostic molecular genetic procedures for this group of rare disorders.
The LeukoDataBase is part of a European effort to set up a transnational database for rare diseases (for more information, please visit the FP7 RD-Connect project website: www.rd-connect.eu).

Regarding biomarkers, the Leukotreat aimed at providing patients with new therapeutic strategies for significantly improving LDs treatment, inexistent today. Indeed, **several pharmacological approaches have been envisaged** using *in vitro* and *in vivo* models of prototypic LD. In a more ambitious approach LeukoTreat project proposed **the development of innovative therapeutic approaches to be translated into clinical trials.** Identification and validation of biomarkers is a prerequisite for successfully carry on clinical trials.
Specifically in this WP2, we first focused on biomarkers **already identified such as NAA** in order to (i) **validate their specificity** taking advantage of the significant samples registered in the LeukoDB (WP1) and (ii) give insight into its **pathophysiological significance** for further therapeutic targets in LDs. As for NAA, studies in vitro indicate that this marker is not toxic per se, but rather appears a consequence of the pathology. Moreover, we have successfully identified novel lipid and oxidative stress biomarkers, which may be of use in monitoring clinical trials in LD patients, for the assessment of innovative therapeutic strategies. A correlation of cause-effect between these markers and disease progression will next be established, and also, their potential role as constituents of specific disease signature and prognosis. Of high value for diagnosis and prognosis of CACH/VWM leukodystrophy, is the set up of a robust method for quantifying eIF2B activity, using recombinant human eIF2B factor produced in yeast, with high potential for use as a routine diagnostic tool.

Regarding pharmacological strategies, funding of the LeukoTreat consortium by the European Community has allowed major progress with respect to current therapeutic concepts for various leukodystrophies, in particular for X-ALD, PMD, and MLD. This includes the systematic screening for molecules with neuroprotective or anti-oxidant/anti-inflammatory activity, molecules that clear misfolded proteins or that enhance protein-translation, and the application to *in vitro* and *in vivo* models of prototypic leukodystrophies.
Some molecules are under final evaluation for the initiation of preclinical/clinical therapy trials. Importantly, the molecular and cellular pathobiology of all leukodystrophies differs in principle. This prevents therapeutic concepts designed for one particular leukodystrophy from being directly applied to a different leukodystrophy. This makes it very valuable that the therapeutic efficacy of antioxidant dietary supplements has now been demonstrated in models of X-ALD and PMD. A related proof-of-concept clinical trial for X-ALD has been initiated, and a human clinical trial for X-ALD has been approved. A proof-of-concept clinical trial for PMD will require an additional prior preclinical trial to test the efficacy of a modified active substance with improved bioavailability. The blood-brain barrier is the major obstacle for enzyme replacement therapy in MLD.
Preclinical and clinical trials are needed to evaluate the benefit of direct enzyme delivery to the cerebrospinal fluid and the therapeutic potential of less invasive strategies using therapeutic enzyme modified to overcome the blood-brain barrier. While LeukoTreat has emphasized the progression of therapy concepts, it also became evident that further understanding of white matter physiology and pathophysiology will be required to develop rational therapy concepts for the majority of other leukodystrophies. Finally, LeukoTreat has fostered the direct cooperation between basic researchers and clinicians, which will be crucial for the success of the consortium’s common aims also after the end of project.

Regarding innovative gene and cell therapies, the proof-of-concept (POC) in mice is now an easy task and can be achieved relatively rapidly. A first challenge is to accelerate the development/translation from POC in mice to clinical trials in LD patients. This requires to evaluate efficiency and safety issues (adverse effects due to overexpression, off-target expression of therapeutic gene) in large animals, in particular non-human primates. This is very costly and requires special platforms. The last challenge is the cost needed to set-up full Good Manufacturing Practice phase I/III trials. This is not specific to LDs and concerns all inherited rare disorders. It requires partnership with the pharmaceutical industry.

Dissemination of LeukoTreat has the originality to be coordinated by the family association devoted to LDs, European Leukodystrophy Association in closed collaboration with France Europe Innovation and the Ethic laboratory of the Paris Descartes University. Develop agreement with partners to deploy the LeukoDB everywhere as well as to maintain the system at the end of the program has also been done.
The total number of publications acknowledging LeukoTreat Grant was 76 at the end of the project. In addition LeukoTreat has been acting as a precursor regarding the Open Access Infrastructure for Research in Europe (OpenAIRE).