Project Final Report

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Name, title and organisation of the scientific representative of the project's coordinator:

Dr Angela Schulz - Universitaetsklinikum Hamburg-Eppendorf

Tel: +49 40 74105 6391

Fax: +49 40 74105 5137

E-mail: an.schulz@uke.de

Project website address: www.dem-child.eu

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DEM-CHILD

Logo:

Project title: **A Treatment-Oriented Research Project of NCL Disorders as a Major Cause of Dementia in Childhood**

Website: **www.dem-child.eu**

Contractors involved (DEM-CHILD consortium):

The project is coordinated by Dr Angela Schulz [CR01] UKE Universitaetsklinikum Hamburg-Eppendorf, Germany, Address of the Coordinator

Universitätsklinikum Hamburg-Eppendorf

Department of Pardiatrics

Martinistrasse 52

20246 Hamburg

Other partners and team leaders:

[CR02] Folkhälsan Samfundet Folkhälsan i svenska Finland rf, Finland

[CR03] UCL University College London, United Kingdom

[CR04] UNIVR Universita Degli Studi di Verona, Italy

[CR05] PGIMER Post Graduate Institute of Medical Education and Research, India

[CR06] imaGenes imaGenes GmbH, Germany

[CR07] ZfS Zentrum für Stoffwechseldiagnose Reutlingen GmbH, Germany

[CR08] KCL King's College London, United Kingdom

[CR09] GSTT Guys and St Thomas' NHS Foundation Trust, United Kingdom

[CR10] GABO:mi GABO:MI GESELLSCHAFT FUR ABLAUFORGANISATION:MILLIARIUM MBH & CO KG, Germany

[CR11] CRM Coastal Research and Management GbR, Germany

[CR12] SBS SourceBioScience, United Kingdom

[CR13] StC Steinbeis GmbH & Co KG, Germany

1.1 Executive summary

The DEM-CHILD project focussed on the main cause for childhood dementia in Europe, the neuronal ceroid lipofuscinoses (NCLs). The NCLs are characterized by visual loss, dementia, epilepsy and progressive physical decline caused by profound neurodegeneration leading to an early death of the patients. Since no cure is currently available, these disorders represent a serious social, medical, and economic challenge.

In order to advance the development of treatment options for the NCL diseases, the DEM-CHILD project has combined the expertise of (i) recognized **European research teams**, both basic scientists and clinicians, (ii) high-technology **SMEs**, (iii) experts in **medical ethics**, and (iv) NCL **patients and family** associations.

This multidisciplinary, scientific and medical interactive European effort to develop innovative therapies in NCLs is based on the pillars of the European Commission Call for proposal HEALTH.2011.2.2.1-4: Creating clinical and molecular tools for experimental therapy of paediatric neurodegenerative disorders causing childhood dementia in Europe and India with the following key objectives:

(1) Development of new testing and screening methods applicable to the wider community

Due to the broad genetic heterogeneity of the NCLs, testing for NCLs is expensive in both time and cost. Therefore, in **WP1** and **WP2**, new time- and cost-effective testing and screening methods for NCL diseases have been developed by three high-technology SMEs.

(2) Prevention and early detection of NCL diseases

WP8 has focussed on this objective by disseminating knowledge about NCL disease and an NCL diagnostic algorithm to increase awareness of these diseases.

As **WP1** and **WP2** have developed innovative cost- and time-reducing testing methods for all NCLs they significantly contribute to an early detection of NCL diseases.

Knowledge about the natural course and clinical spectrum of known and potentially new NCL forms has been gathered in **WP3** and **WP7** and introduced into a new diagnostic algorithm for the NCL diseases.

(3) Studying disease prevalence and incidence

In **WP3**, prevalence figures of each form of NCL have been established in the participating countries.

Estimated birth incidence figures for CLN1, CLN2, and CLN10 are measured in selected areas using targeted population screening supported by automated enzyme analysis in dry blood spots (WP2).

(4) Quantitative description of natural histories

WP3 has implemented a novel network including the most experienced NCL clinicians in Europe and India, to collect the world's largest, clinically, and genetically best characterised, set of natural history data of NCL patients. Significantly, based on this set of detailed quantitative description of natural histories, a tool has successfully been established for the evaluation of future experimental therapies as well as current palliative care treatments.

(5) Characterisation of molecular basis and pathophysiology in relevant models

WP4 and **WP5** have characterised the molecular basis and pathophysiology in models for CLN3 and CLN1 diseases respectively, and have idenitified modifiers and biomarkers of CLN3 and CLN1 diseases.

(6) Development of innovative therapies for NCL diseases

In WP6 the main objective has been to explore the challenges facing the development of therapies for NCL disease caused by mutations in intracellular transmembrane proteins. Juvenile CLN3 disease and CLN6 disease have been chosen because their pathophysiology is already well characterised. These efforts focus upon two complementary strategies: a) using viral-mediated gene transfer to deliver a functional copy of the disease-causing genes; and b) neural stem cell-mediated delivery of neuroprotective factors.

1.2 Summary description of project context and objectives:

Background and Aims

The DEM-CHILD project focused on the main cause for childhood dementia in Europe, the neuronal ceroid lipofuscinoses (NCLs). The NCLs belong to the group of lysosomal storage disorders. They are characterized by visual loss, dementia, epilepsy and progressive physical decline caused by profound neurodegeneration leading to an early death of the patients. The NCLs are the main cause for childhood dementia in Europe, and since no cure is currently available, these disorders represent a serious social, medical, and economic challenge.

In order to advance the development of treatment options for the NCL diseases, the DEM-CHILD project combined the expertise of (i) recognized European research teams, both basic scientists and clinicians, (ii) high-technology SMEs, (iii) experts in medical ethics, and (iv) NCL patients and family associations.

The project implemented a novel network including the most prominent NCL researchers, both basic scientists and clinicians, in Europe, collaborating with Indian experts, to collect the world largest, clinically and genetically best characterised set of NCL patients. Furthermore, we built upon the information generated by the consortium researchers during the last ten years, to identify and characterise novel biomarkers and modifiers, and to develop novel therapies for NCL. The NCLs are defined as lysosomal storage diseases. Amongst all paediatric neurodegenerative disorders, they are especially suitable for development and monitoring of therapies. Excellent cell and animal models exist for this group of diseases, enabling the characterisation of pathological events, screening of disease modifiers and biomarkers required for the development of treatment strategies and monitoring their effects.

Objectives of DEM-CHILD

The multidisciplinary, scientific and medical interactive European effort to develop innovative therapies in NCLs of DEM-CHILD is based on the pillars of the European Commission Call for proposal HEALTH.2011.2.2.1-4: Creating clinical and molecular tools for experimental therapy of paediatric neurodegenerative disorders causing childhood dementia in Europe and India with the following key objectives:

(1) Development of new testing and screening methods applicable to the wider community

Due to the broad genetic heterogeneity of the NCLs, testing for NCLs is expensive in both time and cost. Therefore, in **WP1** and **WP2**, new time- and cost-effective testing and screening methods for NCL diseases will be developed by three high-technology SMEs.

(2) Prevention and early detection of NCL diseases

WP8 will focus on this objective by disseminating knowledge about NCL disease and an NCL diagnostic algorithm to increase awareness of these diseases.

As **WP1** and **WP2** will develop innovative cost- and time-reducing testing methods for all NCLs they will significantly contribute to an early detection of NCL diseases.

Knowledge about the natural course and clinical spectrum of known and potentially new NCL forms will be gathered in **WP3** and **WP7** and be introduced into the diagnostic algorithm for the NCL diseases.

(3) Studying disease prevalence and incidence

In **WP3**, prevalence figures of each form of NCL will be established in the participating countries.

Birth incidence figures for CLN1, CLN2, and CLN10 will be measured in selected areas using automated enzyme analysis in dry blood spots, collected from targeted population screening programs (**WP2**).

(4) Quantitative description of natural histories

WP3 will implement a novel network including the most experienced NCL clinicians in Europe and India, to collect the world's largest, clinically, and genetically best characterised, set of NCL patients. Significantly, a detailed quantitative

description of natural histories will aim at the establishment of an evaluation tool for assessment of future experimental therapies.

(5) Characterisation of molecular basis and pathophysiology in relevant models

WP4 and **WP5** will characterise the molecular basis and pathophysiology in models for CLN3 and CLN1 diseases respectively, aiming at the identification of modifiers and biomarkers of CLN3 and CLN1 diseases.

(6) Development of innovative therapies for NCL diseases

In **WP6** the main objective is to explore the challenges facing the development of therapies for NCL disease caused by mutations in intracellular transmembrane proteins. Juvenile CLN3 disease and CLN6 disease have been chosen because their pathophysiology is already well characterised. These efforts focus upon two complementary strategies: a) using viral-mediated gene transfer to deliver a functional copy of the disease-causing genes; and b) neural stem cell-mediated delivery of neuroprotective factors.

Work strategy and general description

All WPs started at month 1 of the project and worked in parallel closely linked to each other. Three WPs (***WP1***, ***WP2***, ***WP7***) focused on improving the genetic diagnosis of all known and new NCL forms, in order to support early diagnosis and prevention of these disorders. Three WPs (***WP3***, ***WP4***, ***WP5***), demonstrated how the transfer of clinical research on natural disease courses and the clinical spectrum of NCLs (***WP3***) has a direct impact on basic research projects, focussing on the identification of modifiers and biomarkers for NCL diseases. This approach led to the identification of therapeutic targets for experimental therapies. One large, very important WP (***WP6***) solely focussed on the development of experimental therapies for NCLs.

WP1 developed an innovative tool for the diagnosis of all known and novel NCL mutations, including mutations in NCL candidate genes. This NGS-based diagnostic tool significantly reduces the time and costs needed for genetic diagnosis of all NCL forms. In addition, it renders the diagnostic process more precise, because not only coding regions, but also intronic and promoter regions of both, known and NCL candidate genes can be analysed.

In parallel, ***WP2*** developed another diagnostic tool: A multiplex automated enzyme activity testing assay for CLN1, CLN2, and CLN10 disease applying mass spectrometry on dry blood spots. This will allow easy and rapid low-cost diagnosis for these NCL forms worldwide. In addition, this diagnostic tool is used for high-throughput enzyme testing for CLN1, CLN2, and CLN10 disease as part of targeted population screening in order to estimate prevalence and incidence of these types of NCL in selected geographical areas. Diagnostic data and numbers allowing epidemiologic conclusions will be transferred and analysed in ***WP3***. Until WP1 and WP2 had developed these innovative diagnostic tools, patients have already been diagnosed using the traditional, well established techniques such as genetic sequencing and fluorometric enzyme activity assays.

WP3 had three objectives: (i) to measure incidence and prevalence of each type of NCL by collecting, as thoroughly as possible, patients with different NCL forms; (ii) to establish an NCL mutation and NCL patient registry of long-lasting function to describe accurately and in detail the clinical course and clinical spectrum, as well as genotype-phenotype variability in different forms of NCL; and finally (iii) to establish a tool for the evaluation of experimental therapy studies in the NCLs. Once ***WP1*** and ***WP2*** had developed the innovative diagnostic tools and once the knowledge with regard to NCL was disseminated extensively in other countries, e.g. India, in ***WP8***, as expected the number of samples for NCL diagnosis and therefore the number of patients for the registry has significantly increased.

WP4 and ***WP5*** have both focussed on the identification of modifiers and biomarkers of CLN3 and CLN1 disease, respectively. Both WPs utilized established cell lines, patient cell lines and iPS cells from patients. They have employed cell biological and ultrastructural techniques to monitor specific defects in cytoskeletal and mitochondrial structure and function, lipid metabolism and oxidative stress. For biomarker and modifier gene detection, genome-wide technologies, including mRNA and protein profiling, mass-spectrometry-based MALDI-IMS profiling and bioinformatics have been utilised in cell models, as well as in mouse and patient brain tissue.

WP6 has explored complementary therapeutic strategies for the forms of NCL that are caused by mutations in transmembrane proteins CLN3 and CLN6. Due to the nature of these proteins, these increasingly prevalent forms of NCL are particularly difficult therapeutic targets. Focussing our efforts on the eye and brain, as the main pathological targets in these disorders, both (i) viral mediated gene transfer and (ii) stem cell-mediated delivery of neuroprotective factors have been tested.

The goal of ***WP7*** was to identify new NCL genes, as it was clear from family studies, and animal models for NCL, that these must exist. This goal should be accelerated by increasing the available number of families diagnosed with NCL, in particular those arising from consanguineous relations and those having multiple offspring and countries like India are an untapped source for such families.

WP8 has focussed on teaching physicians and medical personnel on diagnostic algorithm, performance and evaluation of procedures for NCL diagnostics. In addition, ***WP8*** has worked on the dissemination of scientific results gained from the DEM-CHILD project.

WP9 had the objective to ensure the proper scientific management of the project in order to strengthen and support the Participants to achieve the objectives, complete the milestones in time and deliver the deliverables as well as to make sure that contractual duties are carried out and intellectual property rights are maintained.

Management structure and procedures

The Project Coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. She submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO:mi she was responsible for the proper use of funds and their transfers to participants. The DEM-CHILD office was established by and based at the coordinator in Hamburg and at GABO:mi in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at GABO:mi was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The General Assembly was in charge of the political and strategic orientation of the project and acted as the arbitration body. It met once a year unless the interest of the project required intermediate meetings. The Steering Committee, the project coordination body consisted of all work package leaders and the Coordinator was in charge of monitoring all activities towards the objective of the project in order to deliver as promised, in due time and in the budget. The Steering Committee met every six months during the funding period. Furthermore, a scientific advisory board and ethical advisory board were implemented to ensure a high standard of research and monitor the progress of the project by taking part in the annual Governing Board Meetings.

1.3 Description of the main S&T results/foregrounds of DEM-CHILD

WP01: Development of a diagnostic gene chip

Objectives

To develop a diagnostic tool to identify known and new mutations in both NCL genes and functional candidate genes, the specific sub-aims being:

To establish a sequence database with all relevant mutations in promoter-, coding- and intronic regions of both NCL genes and functional candidate genes. Public databases for all relevant informations, e.g. splice sites, promoter-regions, etc. will be used to build a profile of relevant mutation loci.

To design a microarray covering promoter-, exon- and intronic regions of all known NCL genes and NCL candidate genes.

To test and optimise the developed microarray as a tool for diagnosing known and new mutations in NCL genes and candidate genes.

To update and complete the diagnostic gene chip as novel NCL-associated mutations and as potential novel NCL genes are identified, to allow the usage of the chip for large-scale diagnostic studies. Extensive hybridisation experiments will be performed to optimise and test the prototype array.

If applicable, extend the chip-based diagnostic tool to next-generation-sequencing technology

The aim of WP01 was to develop a new diagnostic tool for the genetic diagnosis of NCL diseases. This tool should be innovative, reliable, and time- and cost-effective in order to significantly contribute to an early diagnosis of NCLs.

According to the above listed objectives, several steps had to be undertaken during this development process:

Establishment of sequence database

In preparation of the diagnostic chip design, it was necessary to establish a sequence database with all available information about known or putative NCL-relevant mutations. Since the DEM-CHILD grant application in 2011, the number of known NCL genes had increased from eight to at least thirteen (Warrier V et al. BBA 2013, Schulz A et al. BBA 2013). In consequence, the identification of new NCL genes had broadened the number of putative NCL candidate genes from nine to more than twenty. During several WP01 workshops and phone conferences, all geneticists involved in the DEM-CHILD project developed and agreed on a most up-to-date list of thirteen NCL genes and twenty-five NCL candidate genes. Together with project partner 03 (Sara Mole, UCL), who runs the NCL resource database, and the partners 01, 02, and 04, the list of relevant mutations was agreed and respective sources of information were identified and imported to our local server.

For the project partners, an excerpt of the database was provided as flat file in dynamic excel format, which allowed direct linking-out to external information systems like the UCSC genome browser. An example is given below in Figure 1. To date, the database contains 38 gene loci, which cover about 1.5 Mb (repetitive elements not counted) of sequence. Apart from the 13 genes carrying known NCL-related mutations, we added 25 more genes, which are of potential disease relevance as they cause NCL resembling disease in animals or NCL-like phenotypes in patients or represent good functional candidates for the disease. In total, these are 21 more genes than originally planned for the chip reflecting the rapidly growing field of NCL diseases.

Type	mutations	accession genomic locus (RefSeq)	gene per UCSC	GWAS Central (previously the Human Genome Variation database	chrom	start	end	region	orientation	UCSC gene locus (link opens UCSC genome browser at gene locus)
cln1	70	NG_009192.1	PPT1	HGV-db: PPT1	1	40537382	40564142	26761	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln2	122	NG_008653	TPP1	HGV-db: TPP1	11	6640693	6633996	6698	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln3	70	NG_008654.1	CLN3	HGV-db: CLN3	16	28488600	28503623	15024	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln4	2	NG_029805	DNAJC5	no entry in HGV	20	62525455	62568384	42930	for	http://genome.ucsc.edu/cgi-bin/hgTracks
cln5	71	NG_009064.1	CLN5	no entry in HGV	13	77565059	77577652	12594	for	http://genome.ucsc.edu/cgi-bin/hgTracks
cln6	70	NG_008764	CLN6	no entry in HGV	15	68550444	68498330	52115	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln7	49	NG_008657	MFSD8	no entry in HGV	4	128888139	128838959	49181	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln8	24	NG_008656	CLN8	no entry in HGV	8	1711870	1734736	22867	for	http://genome.ucsc.edu/cgi-bin/hgTracks
cln10	10	NG_008655	CTSD	no entry in HGV	11	1772982	1786222	13241	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln11	2	NG_007886	GRN	no entry in HGV	17	42422491	42430470	7980	for	http://genome.ucsc.edu/cgi-bin/hgTracks
cln12	1	NG_009054	ATP13A2	HGV-db: ATP13A2	1	17312453	17338423	25971	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln13	5	NG_013304	CTSF	HGV-db: CTSF	11	66330935	66336047	5113	rev	http://genome.ucsc.edu/cgi-bin/hgTracks
cln14	3	NG_028110	KCTD7	HGV-db: KCTD7	7	66093868	66108216	14349	for	http://genome.ucsc.edu/cgi-bin/hgTracks

Fig.1: The NCL candidates sequence mutation database, version 1, which is basis for the first generation of the diagnostic chip. The links open access to all kinds of information needed for the array design, plus useful information concerning gene loci and annotations

Design and update of a microarray covering promoter-, exon- and intronic regions of all NCL genes and NCL candidate genes

The first test version of the microarray-based diagnostic chip was produced by Agilent and covered all thirteen known NCL genes including exons, introns and about 3 kb promoter regions. The original plan was to use Nimblegen arrays, but since the announcement of Roche in early 2012 to close down the Nimblegen array production, we had to employ an alternative system. Agilent offered the same capacities for a very comparable price and could be processed by partner 06 (imaGenes in Berlin), as well. As this version was used as a primary test version for positive control samples, NCL candidate genes were not yet included for cost-effective reasons.

For **the second version** of the chip, apart from the 13 genes carrying known NCL-related mutations, we added 25 more NCL candidate genes, which are of potential disease relevance as they cause NCL resembling disease in animals or NCL-like phenotypes in patients e.g. genes associated with myoclonic epilepsies (PME). Those have been added as a result of the recent finding that *KCTD7*, a gene causative for PME type 3, might also be responsible for an infantile NCL phenotype, termed CLN14 (Staropoli J et al. Am J Hum Genet 2012).

Testing of the developed microarray as a tool for diagnosing known and new mutations in NCL genes and candidate genes

A total of 32 positive control samples of NCL patients with known NCL mutations were provided by partners 01, 02, 03, and 04. Samples were selected to cover almost all known NCL genes and to provide a mixture of compound heterozygous and homozygous mutations as well as a mixture of point mutations, insertions and deletions. All samples were blinded at the coordinator's site, received a unique identifier code, and were entered into the sample database. After being sent to partner 06, they were prepared in parallel and used for hybridisations on the first test version of the diagnostic chip.

The testing procedure included several steps in the microarray laboratory, with modest technical changes as compared to the standard Agilent CGH protocol, i.e. hybridisation times were increased by 4 hrs (= 16% of standard time). No significant changes were observed in the final intensities per oligo and in the final analysis results.

Testing of the microarray-base diagnostic chip revealed two major issues of this technique:

(i) While the diagnostic chip was able to pinpoint exonic regions where the disease-associated mutation is known to reside in approximately 50% of the samples, for the remaining approximately 50% of samples, the interval with the known mutations were not clearly identified by the diagnostic chip. Possible explanations included poor DNA quality/quantity, hybridisation problems, or the need for optimisation of the chip design.

ii) In addition to potential known NCL mutations, the chip apparently identified a significant number of gene regions being different between patient samples and reference (commercially available Promega male genomic DNA), the majority of which were not known before, as in most cases previous studies have been limited to one or a few NCL genes.

Both issues required further investigation and had significant influence on the task to test and optimize a new time- and cost-effective method for genetic diagnosis. In the meantime, since our initial working plan, technology had further developed and made customized targeted gene deep-sequencing in next-generation more affordable and practicable in neurogenetic laboratories. In particular, it seemed now cost-effective to combine the speed of PCR with the sensitivity of hybridization providing a robust solution for targeting smaller capture regions such as it occurs in the analysis of a limited set of genes.

Change of core technology from microarrays to Next-Generation-Sequencing (NGS)

After discussion between the project coordinator and partners 1, 2, 4, 6, 11, 12 there was a clear decision to go on with the NGS approach. As expected, analysis of the first batch of 20 positive control samples from patients with known NCL mutations by NGS showed that the sequencing information was more precise and allowed deeper analysis than the microarray-based data. Moreover, per sample the total costs of the NGS approach could be reliably estimated to be almost equivalent to the array costs as of September 2013. The conclusion was to save budget and further consumables for tests and improvement of the diagnostic chip, and in turn to continue 100% with the development and set-up of the diagnostic sequencing tool taking the following steps:

a) Target enrichment system development

Partners 11 and 12 designed a novel tool for target enrichment and carried out a series of tests to evaluate the potential of the NGS-based diagnostic tool.

b) Test sequencing and quality analysis of the HaloPlex system

16 positive control samples from partners 1, 2, 3, 4, most of them already used for the microarray testings, were taken for the initial tests of the target enrichment step. All sample preparations, processing of the customized Agilent HaloPlex kit and the sequencing runs on an Illumina MiSeq machine were done by partner 12. Partner 11 developed a tailored Bioinformatics pipeline to map the millions of reads to the 39 gene loci and to perform all statistical and mutation analysis steps. Since the goal of the NGS diagnostic tool was to become a routine application in NCL diagnostics it was of major importance that the target enrichment delivers reproducible results which could be shown by comparing several testings of the same samples. In conclusion, the target enrichment system provided sufficient quality for routine applications in NGS based diagnostics of NCL.

c) Development of Bioinformatics package for an NGS-based diagnostic tool

The change of strategy from a microarray-based analysis system to an NGS-based approach required development of a completely new software package. This was carried out by partner 11 in the second half of DEM-CHILD. By the end of the project, the software package was completed and can be applied in routine analysis projects now.

The package was designed to run fast and efficiently. Even large numbers of samples can be processed on standard servers in a reasonable time frame

All annotated variations as they were published by the 1000 genome project and other large genome projects were downloaded and are used to flag mutation candidates accordingly. This feature allows filtering of likely polymorphic variants against NCL specific mutations.

In the final step the software was extended by modules to compare single sample analyses against each other. This is very useful, since it allows ranking of mutation candidates in replicate analysis runs, which is the optimal mode of NGS-based analysis. Partner 11 and 12 are open to offer the complete NCL diagnostic tool as a routine service to partners and researchers in the NCL field.

d) **Final evaluation of NGS diagnostic tool in large numbers of samples**

In the last stage of the DEM-CHILD project partners 1,2,3,4,5 collected DNA from altogether 209 NCL samples, which were to a large extent run in duplicates. The quality of the found mutations was extensively evaluated and adjusted in a large number (16) of reference samples, where NCL-related mutations were already mapped by PCR-screenings or classical Sanger sequencing. Almost all mutations were correctly mapped, in one case the NGS-based diagnostic tool could even improve the previous analysis: A mutation denoted as ‘homozygous’ could be corrected into heterozygous by the new tool.

Conclusion

WP1 has successfully developed a novel NGS-based tool for the diagnosis of all known and novel NCL mutations, including mutations in NCL candidate genes. This tool reduces the time and costs needed for genetic diagnosis of all NCL forms significantly and can now be offered to the public as diagnostic service.

WP02: Development of automated enzyme testing for CLN1, CLN2, and CLN10

Objectives

To establish an automated enzyme testing methodology for CLN1, CLN2, and CLN10 diseases in dry blood spots using tandem mass spectrometry, in order to obtain a tool for easy rapid low-cost diagnosis worldwide.

To perform cost-effective high-throughput enzyme testing for CLN1, CLN2, and CLN10 diseases in order to estimate prevalence and measure birth incidence of these types of NCL in selected geographical areas.

Establishment of an automated enzyme testing methodology for CLN1, CLN2, and CLN10 diseases in dry blood spots using tandem mass spectrometry

Transfer of existing enzymatic fluorometric assays to tandem mass spectrometry (TMS)

At the beginning of the project, enzymatic fluorometric assays for determination of palmitoyl protein thioesterase 1 (PPT1 / CLN1), tripeptidyl peptidase 1 (TPP1 / CLN2) and cathepsin D (CLN10) in dried blood spots had already been established by partner 01 and were regularly used in the NCL diagnostic service of partner 01 (Lukacs et al., 2003). In order to establish an automated enzyme testing methodology in the laboratory of SME partner 07, this technique was transferred to tandem mass spectrometry (TMS).

The enzymatic activity assays for CLN1 and CLN2 were carried out according to the procedures published by Lukacs et al. The sample workup was altered due to the requirements for tandem mass spectrometry. For quality control, dry blood spot samples derived from patients with genetically confirmed diagnosis of CLN1 or CLN2, respectively, were used. Samples were tested in parallel by partner 07 using the newly developed TMS-based method as well as by partner 01 using the original fluorometric assay. Table 1 gives an overview of the results obtained by each method. At a qualitative scale, all patients could be clearly assigned to their respective disease using TMS. However, quantitative data differed between the two methods. TMS and fluorescence assays both lead to the same results in respect to the diagnosis of CLN1 or CLN2 deficiency. Affected patients could be clearly distinguished from not affected persons or healthy carriers.

Table 1: Quantitative data of known patients affected with CLN1 or CLN2 (LOD=Limit of Detection)

	PPT1 (CLN1)		TTP1 (CLN2)	
Patient /Disease	TMS [nmol/spot/48h]	Fluorometric [nmol/spot/45h]	TMS [nmol/spot/48h]	Fluorometric [nmol/spot/45h]
#1 / CLN1 Disease infantile	< LOD	0.0	1.30	0.3
#2 / CLN1 Disease juvenile	< LOD	0.01	1.03	0.26
#3 / CLN2 Disease late infantile	3.6	1.20	< LOD	0.01
#4 / CLN2 Disease late-infantile	2.0	0.9	< LOD	0.0
#5 / CLN1 Disease infantile	< LOD	0.01	1.10	0.32

When attempting to transfer enzymatic fluorometric assays to tandem mass spectrometry (TMS), SME partner 07 came across several challenges:

i) No method for fluorometric measurement of cathepsin D (CLN10) activity in dried blood spot was published in the literature so far. During the first reporting period, partner 07 did not succeed in establishing a selective method using two different already commercially available substrates and different assay conditions. None of the methods could clearly distinguish between positive and negative controls, e.g. wild type and cathepsin D knock out mice. Moreover, gathering samples from patients with cathepsin D deficiency was difficult, due to the fact of the rareness of this specific disease and of the early death within weeks after birth of most affected patients. Affected and unaffected mice showed higher amounts of reaction product in comparison to healthy human samples, which may be justified with the different metabolisms.

ii) Use of the original fluorometric substrates in mass-spectrometry-based assays required frequent cost- und time-intensive cleaning of the mass spectrometer which increased diagnostic costs per sample significantly.

Consequently, costs for the initially intended screening of a high number of anonymous dry blood spots collected from newborn-screening programs became too high (>200 000 €).

Taken together, these problems led to the following **change in the work program** for the current reporting period which had been agreed with the EC scientific officer as **part of amendment No. 2** of the original DoW:

The consortium agreed that the use of completely newly developed substrates for all three mass-spectrometry based enzyme activity assays (CLN1, CLN2, and CLN10) was necessary. This led to the addition of partner 13 (StC) to the consortium who recently had successfully developed such substrates for CLN1, CLN2, and CLN10 enzyme activity assays which are suitable for both – fluorometric and MS-based assays - without requiring required frequent cost- und time-intensive cleaning of the mass spectrometer. Moreover, the consortium agreed, that the successful application of these new substrates should lead to the development of a MS-based triplex enzyme assay allowing measurement of enzyme activities of CLN1, CLN2, and CLN10 in the same assay. This would render the assay more time- and cost-effective.

Development of a MS-based multiplex array for CLN1, CLN2 and CLN10

The development of a MS-based specific and highly sensitive clinical diagnostics triplex assay for CLN1, CLN2, and CLN10 (cathepsin D) in dry blood spots (DBS) was successfully performed taking several steps:

1. Successful adaptation of newly developed specific substrates based as internal standards suitable for triplex assay

2. Mass spectrometric (MS-MRM) single determinations of enzyme activities, validation by fluorimetric determination

3. Development of a mass spectrometric triplex assay for CLN1, CLN2, CLN10 by MS-MRM using the new substrate derivatives

4. Evaluation of triplex assay by determination of positive control patient samples (Fig. 2)

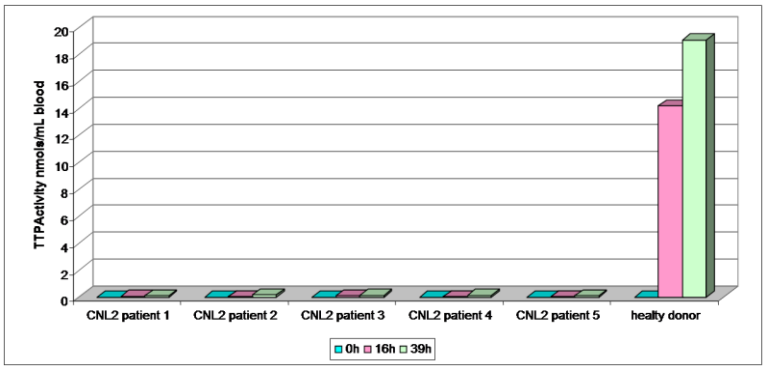


Fig. 2. Tripeptidyl peptidase activity determined by MS-MRM for CLN2 patients and healthy donors.

Performance of cost-effective high-throughput enzyme testing for CLN1, CLN2, and CLN10 diseases in order to estimate prevalence and measure birth incidence of these types of NCL in selected geographical areas

During the first reporting period, the original approach to measure birth incidence figures for specific NCL forms (CLN1, CLN2, CLN10) was to screen a high number of anonymous dry blood spots collected from newborn-screening programs. As the use of the original fluorometric substrates in mass-spectrometry-based assays required frequent cost- und time-intensive cleaning of the mass spectrometer the diagnostic costs per sample increased significantly. Consequently, costs for the initially intended screening of a high number of anonymous dry blood spots collected from newborn-screening programs became too high (>200 000 €). These problems led to the following change in the work program agreed with the EC scientific officer as part of amendment No. 2:

Instead of using a large number of anonymous dry blood spots, the consortium suggested to perform targeted population screening in order to estimate incidence figures of CLN1, CLN2, and CLN10 disease: Due to the above mentioned challenges and the necessity to include a new partner into the consortium, the new triplex enzyme assay using the new substrates had only been available since end of month 35 of the project. Therefore statistical numbers from targeted population screening are still under way. Further results are explained in WP03.

Conclusion

In **WP2,** a mass-spectrometry based multiplex enzyme activity assay has successfully been developed which is able to measure enzyme activities for CLN1, CLN2, and CLN10 disease in parallel.

WP03 Epidemiology and Natural History Study

Objectives

To collect, as thoroughly as possible, patients with different NCL forms in all participating countries including India in order to measure the incidence and prevalence of each type of NCL

To establish an NCL patient registry of long-lasting function to enable both retrospective and prospective patient data collection and to precisely describe the clinical course and its variability in the different forms of NCL

To correlate the genotype of patients with their phenotype by linking clinical and genetic mutation data

To establish a tool for evaluation of future experimental therapy studies, as well as current palliative therapies

Collection of patients with different NCL forms in all participating countries to measure incidence and prevalence of each type of NCL

Prevalence figures

The first aim of the epidemiology task was to establish prevalence figures in the participating countries (Germany, Italy, Finland, UK and India). As the precision of these figures clearly depends on i) the awareness of NCL diseases, ii) existing medical infrastructure, and iii) diagnostic capabilities in the respective areas, the DEM-CHILD consortium has worked towards optimising diagnostic capabilities in the participating countries by taking several steps:

i) Increasing awareness of NCL diseases

Multiple actions have been undertaken in order to increase the awareness of NCL diseases across different medical specialties such as paediatricians, child neurologists, neurologists, ophthalmologists, and geneticists. Numerous members of the DEM-CHILD consortium have given talks about the DEM-CHILD project and NCL diseases both at national and international scientific meetings addressing physicians from different medical specialties (see list of dissemination activities). In addition, six DEM-CHILD teaching workshops have been organised in several countries including India. In addition, several review articles on the clinical presentation of NCL diseases have been published by DEM-CHILD partners during the reporting period. Moreover, the project website www.dem-child.eu provides up-to-date information and useful links for clinicians who would like to learn more about these diseases.

ii) Development and distribution of a diagnostic algorithm

A diagnostic algorithm for NCL diseases has been developed and published in collaboration with all NCL clinical experts in the consortium as part of a review on clinical presentation of NCL diseases (Schulz A. et al, BBA 2013). A flyer illustrating both, this diagnostic algorithm for NCL diseases as well as providing information where to send diagnostic samples, has been designed and distributed not only during the respective DEM-CHILD teaching seminaries but also at various international congresses (see list of dissemination activities). The flyer can also be downloaded for free from the DEM-CHILD website.

iii) Optimization of NCL-diagnostic workflow

Several steps have been taken in order to optimise the diagnostic workflow for NCL diseases:

a) 24h expert advice: Experts from the NCL specialty clinic of co-ordinator partner 1 can be reached 24 h/ 7 days by phone (+49-40-74105-6391) and discuss patient cases with suspected NCL in order to be recommended on diagnostic procedures to be performed.

b) Centralised sample database: Once NCL disease is clearly suspected in a patient, physicians are advised to refer their patient's samples (DBS, DNA; fibroblasts etc.) for diagnosis to the project. Information regarding where and under which conditions diagnostic samples should be sent can be found on the project's website. The diagnostic workflow developed by the DEM-CHILD consortium recommends that all diagnostic samples should first be sent to partner 01, where each sample receives a unique identifying code and the respective patient's information such as age at disease onset, consanguinity etc. is entered into a sample database under this code. The project-specific patient sample database was developed by partner 01 in order to code and track samples referred to the DEM-CHILD project. The database is online accessible and password protected. Geneticists and physicians who have

referred samples to the project will receive their samples' unique codes as well as a username and password to login into the sample database and to track their patient's diagnostic results.

The sample database currently holds information on 363 samples from patients with clinically clearly suspected NCL disease (DNA n=278; DBS n=60; skin biopsies / fibroblasts n=25).

iv) Collaboration with NCL family associations

Throughout the entire reporting period, the DEM-CHILD consortium has closely collaborated with the national NCL family associations in the participating countries: Clinical experts have given talks at the annual meeting of the Italian, German and UK family associations meetings. The above mentioned 24 h expert advice phone number is offered not only to referring physicians but also to families affected by NCL disease. As part of this expert advice, newly diagnosed patient have been provided with contact information of the respective national family association. Families without a clear genetic diagnosis have been referred to the consortium by the family associations. Moreover, numbers of total patients with NCL disease have been regularly exchanged between the consortium and the family associations in order to establish prevalence figures.

By implementing the step described above throughout the entire project, **prevalence figures for CLN1, CLN2, CLN3, CLN5, CLN6, CLN7, CLN8, CLN10 have been successfully obtained for Germany, UK, Italy and Finland.**

However, one major challenge was to obtain reliable prevalence figures for India:

Due to the late joining of our Indian partner in month 13 of the project and due to a restriction that only 10% of patient material was allowed to leave India and to be entered into the project, the number of samples (n=63) received during the entire project is too small to be used for any prevalence studies. The 10% restriction rule had been enforced by the Indian Council of Medical Research (ICMR) after the project started and was a pre-condition that partner 05 got permission from the ICMR to sign the DEM-CHILD grant agreement and be part of the consortium. The new restriction rule for India had been included in Amendment No. 1 of the DoW and accepted by the European Commission.

Incidence figures

As already pointed out under WP02, as per amendment No.2 of the DoW the work programme to obtain incidence figures for specific NCL forms (CLN1, CLN2, CLN10) has been changed from screening of anonymously collected dry blood spot samples derived from newborn screening programs to targeted population screening in selected areas.

Due to the mentioned challenges developing MS-based enzyme assays for CLN1, CLN2, and CLN10 and the necessity to include a new partner into the consortium, the new MS-based triplex enzyme assay has only been available since end of month 35 of the project. Therefore statistical numbers from targeted population screening are still under way.

Despite these problems, the project has well prepared the basis for targeted population screening by clearly defining the target patient groups: These groups comprise patients who present with "risk factors" for CLN1, CLN2 or CLN10 disease, respectively. These "risk factors" are early, sometimes pre-clinical symptoms which have been shown to occur with high frequency in the respective NCL disease. Risk factors have been identified by extensive analysis of natural history data in the patient database.

In conclusion, studies on epidemiology of NCL diseases in the participating countries have been successful especially with regard to the establishment of up-to-date prevalence figures. As expected, the generation of incidence figures has been a challenge but solutions have been generated.

Establishment of an NCL patient registry for both retrospective and prospective patient data collection

Development of an NCL patient registry has been performed by the DEM-CHILD Patient Database Consortium. To summarise, the DEM-CHILD NCL patient database has the following main features:

1. Online accessible (in compliance with international data safety rules)
2. Patient management software (to avoid inadvertent double or multiple entries of the same patient)
3. Statistical analysis features (to allow immediate comparison of result of one patient to other patients)
4. Database charter (ensuring rules for transparency, data ownership and publication)

The type of data in the database is differentiated into "static" and "dynamic" data.

Patient recruitment

The intended patient recruitment rates were 200 NCL patients in total for this project. All patients had to have a clear genetically verified diagnosis of NCL disease. Due to the low number of diagnosed patients in India, data collection was restricted for this country. Still the intended recruitment rates have been well achieved both for retrospective data analysis (n=220; Table 2) and for prospective data analysis (n= 202; Table 3). Moreover, the database consortium has expanded beyond the DEM-CHILD partners with currently 12 countries participating allowing the database to be the world largest collection of NCL patient data to date (for further details please refer to section 1.3).

Type of data collected

Data collected in the DEM-CHILD database are separated into "static" and "dynamic" data. Static data are e.g. genetic diagnosis, age at first symptoms etc. They have in common that they do not change over time. Dynamic data are related to the patient's age and change with the progression of the disease. Examples for dynamic data are clinical scoring data.

i)"Static" data

Static data do not change over time. They are mainly derived from retrospective data collections based on information from patient charts and detailed interviews with parents and caregivers. Static data collected in the database comprise the following categories: Gender, family history, perinatal history, psychomotor development, medical history, diagnostic summary, neurologic findings, experimental therapy studies. Information collected under the category diagnostic summary do not only comprise the genetic diagnosis, results of enzyme activity assays or electron microscopy but also whether and where (in which participating center) patient material is available such as DNA, fibroblast cell lines etc. This information creates a kind of **"virtual biobank"** which allows scientists to search for patient material with specific mutations for certain research project. Once a patient of whom material is available is identified, the scientist can contact the participating center and ask for permission to use the material for the respective research project. During the DEM-CHILD project, this "virtual biobank" has been well in use e.g. for the identification of specific CLN1 patient fibroblast cell lines for WP05. Table 2 provides an overview on numbers of patients with static data sets for each NCL form segregated by different ages of disease onset (infantile, late infantile, juvenile, adult) and shows that intended recruitment rate has been well achieved.

The static data sets represent the background for the analysis of the dynamic data. They allow the interpretation of different disease progression rate based on the genetic diagnosis and thereby lead to a better understanding of the genotype-phenotype correlation.

In addition, detailed analysis of the retrospective static data has led to the identification of early symptoms supporting early diagnosis.

Table 2: Number of patients with static data (from retrospective data analysis)

NCL Form	infantile	Late infantile	juvenile	adult	Total
CLN1	19	5	3	0	27
CLN2	0	79	2	0	81
CLN3	0	0	72	0	72
CLN5	0	8	4	0	12
CLN6	0	14	0	0	14
CLN7	0	6	0	0	6
CLN8	0	8	0	0	8
Total	19	120	81	0	220

ii) Dynamic data

Dynamic data are related to the patient's age at the time of examination. They will change as the disease progresses. Dynamic data collected in the database comprise the following: Clinical status, neurologic status, current medication, ophthalmologic exam, cardiologic exam, EEG, brain imaging (MRI/MRS). In addition, clinical scoring systems have been regularly used for each patient such as the late infantile NCL Scoring (Steinfeld et al. 2002), juvenile NCL scoring (Kohlschütter et al. 1988), juvenile UBDRS scoring (Marshall et al. 2005), GMFCS (Gross Motor Function Classification System), BFMF (Bimanual Fine Motor Function), and QoL (Quality of Life) Questionnaires. The intended schedule for regular follow-up examination had been every 6 months for patients with infantile and late infantile phenotype and every 6-12 months of patients with a juvenile phenotype. Table 3 provides an overview of the

number of patients and exams collected per NCL form showing that the intended recruitment rate has been well achieved.

Table 3: Number of patients with dynamic data (from prospective data analysis)

NCL Form	Cardiologic	EEG	Ophthalmologic	cMRI		No. Patients with Clinical Scoring
				No Exams	No. Patients	
CLN1	10	22	13	6	3	27
CLN2	31	47	28	26	10	62
CLN3	33	25	52	50	20	79
CLN5	5	13	9	8	8	12
CLN6	6	11	11	6	6	11
CLN7	1	5	11	5	3	5
CLN8	8	3	4	4	4	6
Total	94	126	121	119	52	202

Analysis of genotype-phenotype variability in each NCL form

The analysis of genotype-phenotype variability in NCLs is challenging due to the heterogeneity of these diseases with at least 13 different affected genes and multiple mutations identified in each gene.

Extensive analysis on genotype-phenotype variability has been performed based the data collected in the database. Prospective longitudinal data on clinical scoring data, MRI-based brain volumetric analysis etc. have been evaluated based on static retrospective data such as genetic diagnosis etc. The following are examples for such analyses:

Clinical Scoring in CLN2 disease

For the analysis of natural history of CLN2 disease, the late infantile NCL scoring system established by Steinfeld et al. 2002, has been used in a total of 62 patients with genetically diagnosed CLN2 disease. Scoring data have been collected longitudinally for all patients from birth to the age of 10-12 years. Disease progression was measured longitudinally by the sums of the 3-point motor and language subscales of the Steinfeld et al. score. Disease progression was very similar in the majority of patients. Slowly progressing patients were uncommon and mostly related to unusual genotypes.

(Schulz A et al. The Natural History of Late Infantile CLN2 Disease: Striking Homogeneity of Clinical Progression in Two Independently Obtained Large Clinical Cohorts. Manuscript in preparation).

Brain volumetric analysis in CLN2 disease

In addition to clinical scoring, we have used longitudinal MRI-based brain volumetric studies to study disease progression and genotype-phenotype correlations in CLN2 disease. In this study, twenty-one MRIs of eight patients (3 male; 5 female; mean age, 6.9 ± 2.5 years) with genetically confirmed CLN2 disease were performed. Results showed that longitudinal MRI volumetry of gray matter reveals a unique disease progression. Morever, volumetric data were more sensitive in describing disease progression in later stages.

(*Loebel U. et al. Longitudinal MRI Volumetry of Gray Matter in Neuronal Ceroid Lipofuscinosis Subtype CLN2 Reveals a Unique Uniformity of Disease Progression. Manuscript submitted).*

Genotype-phenotype correlation in CLN3 disease

Similar to CLN2 disease, many different mutations are described for CLN3 disease (n=65). However, literature shows that the majority of patients (up to 80%) harbor a common 1 kb deletion (c.462-677del) which leads to the loss of exons 7 and 8. A total of 58 patients (89%) were homozygous for this mutation in the CLN3 patient cohort analysed from the DEM-CHILD database.

When using a clinical scoring system developed by Kohlschütter et al. for juvenile NCL in all CLN3 patients homozygous for the 1 kb deletion in our cohort, we could detect a rather high phenotype-genotype variability which supports the data previously published by Lebrun et al. When calculating the “Score of Relative Disease Severity” (method described by Lebrun et al. 2011), these patients segregated into three groups with slow (24%), average 36%) and rapid disease progression (40%) despite the fact that they harbor the same mutation in the *CLN3* gene.

Clinical scoring in CLN5 disease

CLN5 disease is very heterogenic with currently at least 45 mutations identified in a total of 85 patients so far according to the DEM-CHILD mutation database. The phenotype observed in CLN5 patients predominantly from Italy

was a mixture of late infantile / early juvenile. Therefore the current clinical scoring systems were not optimal in order to describe the disease in these patients,

Because of the domains which were observed to be affected at the disease onset, an ad hoc evaluation scale was developed by Simonati et al. which is modified from the late infantile Steinfeld et al. scoring. A total of 18 patients with CLN5 disease has been analysed with this new clinical scoring system.

(Simonati A. et al. Analysis of genotype-phenotype variability in Italian CLN5 patients. Manuscript in preparation)

Evaluation tool for (future) experimental studies

To date the DEM-CHILD NCL patient database contains the largest set of natural history data for all NCL forms worldwide which will continue to grow with the participation of the new database consortium members from a total of 12 countries. Results from the analysis longitudinal natural history data describe well that data derived from the DEM-CHILD NCL patient database represent a valid tool for the evaluation of current and future experimental therapy studies. In fact, longitudinal natural history data of 62 patients with CLN2 disease have been combined with a set of cross-sectional data from a total of 43 CLN2 patients seen at the Weill-Cornell-Medical-College by Dr. Ron Crystal. The level of loss of motor and language function over time was similar in both cohorts. In fact, these combined CLN2 natural history data are already used as control data in a phase I/II clinical trial on intraventricular enzyme replacement therapy in CLN2 disease.

Evaluation tool for current palliative therapies

As to date there is still no cure for any form of NCL, palliative treatment and supportive care are an important part of current treatment in all NCL forms.

Therefore information on current medication to treat the most severe symptoms in all NCL forms such as epilepsy, myoclonus and spasticity has been collected in the DEM-CHILD database including the respective positive and negative effects. First results have been published already in a review on the clinical presentation of NCL diseases (Schulz A. et al, BBA 2013) More detailed results are summarised in a review about this study in the Journal of Pediatric Epilepsy which is in press (Kohlschütter et al., J Pediatr. Epilepsy 2014). These data will help to improve palliative medical care for NCL patients.

Conclusion

In **WP3**, prevalence figures for the different NCL forms have been obtained in the participating countries. For incidence studies, a new approach has been implemented by defining criteria such as early symptoms for targeted population screening. An online NCL patient database has been established containing both retrospective and prospective data from over 200 NCL patient to date. Extensive analysis on genotype-phenotype variability has been performed based on longitudinal collection of natural history data. These datasets represent invaluable tools for the evaluation of experimental therapy studies and are already used as control data in clinical trials. Likewise, current palliative medical treatment options have been evaluated based on these data in order to improve palliative care in NCL patients.

WP 04 – Biomarkers and modifiers of CLN3

Objectives

To provide novel biomarkers of CLN3 disease by analysing using genetic high-throughput screening and genome-wide microarray approaches

- a simple well-studied and genetically tractable fission yeast model for CLN3 disease
- homozygous mouse neuronal precursor *Cln3*$^{\Delta ex7/8}$ cell lines
- *CLN3*-depleted cells

To identify and exploit

- conserved pathways that ameliorate the effect of mutations in *btn1* (the homologous yeast gene to CLN3)
- dysregulated potential biomarker genes in *Cln3*$^{\Delta ex7/8}$ neurons
- modifier genes differently expressed during acute down-regulation of CLN3 modelling rapid course alterations

Yeast model for CLN3 disease

Partner 3 has shown that a simple fission yeast model for CLN3 disease can be used to predict the severity of disease caused by different mutations in *CLN3*, consistent with identified metabolic changes and diverse phenotypic effects. The basis for these effects may be alterations that physically distort the Golgi apparatus and affect many downstream pathways. The ease of genetic manipulation in this organism makes this model unique in terms of the potential to identify novel pathways that ameliorate the effects of mutations in *CLN3*.

Integrated yeast strains carrying mutations equivalent to the common juvenile CLN3 disease 1 kb (∆*ex7/8*) deletion or p.Glu295Lys, a mutation associated with a particularly slow disease progression have been successfully created.

The strains have been characterized in terms of marker phenotypes exhibited by a strain completely deleted for btn1.

In order to use these strains in any screens to identify genes that rescue btn1 mutant phenotype(s) or act in the same pathway as btn1 mutant proteins, conditions needed to be identified that robustly differentiate between the growth of wild-type yeast, btn1del, and the mutant strains. This had been successfully achieved.

Identification of genes rescuing btn1 mutant phenotypes in yeast

Towards a high-thoughput screen, conditions were identified that robustly differentiate between the growth of wild-type yeast and *btn1*del strains. A screen of over 1000 FDA-approved small molecules was performed. Three molecules showed efficacy in the yeast disease model, in mammalian cells in vitro, and in a zebrafish morpholino model of CLN3 disease.

A novel transposon mutagenesis approach has been established as a sophisticated method that also allows more comprehensively to identify the genetic interactions of btn1. The method allowed mutagenesis of a yeast culture by making insertions of a modified transposon at a random position in the genome, generally at a frequency of one insertion per cell. This tool is sufficiently well-developed that it is routine to create a library with an estimated 10^9 insertion-mutated cells. This method has been applied to investigate the genetic interactions of btn1.

Six libraries in wild type and cells lacking btn1, have been generated, sequenced, with >23 million good quality reads obtained at an average of 170 reads/kb. Bioinformatic analysis, which is complex, is still being optimized, but it is clear that further optimisation of the method is possible.

Synthetic genetic arrays (SGAs) are a well established tool that can be used to systematically examine the consequences of loss of every gene in a query disease strain, highlighting genetic interactors of the disease gene. Those that enhance fitness are considered positive interactors (genetic suppressors) and those that decrease fitness are negative interactors (synthetically lethal or sick). Positive interactors can provide novel therapeutic candidates, and overall SGAs reveal where the query gene fits within the network of cellular pathways. We performed SGAs in four independent *btn1*∆ isolates from two different backgounds that highlighted 288 positive and 119 negative interactors (Figure 3).

To conclude, genes and small molecules that ameliorate CLN3 disease in the yeast model that can be followed up translationally have been successfully identified. A manuscript is in preparation:

Bond M et al. Genome-wide analysis reveals a central role for Tor signalling in a yeast model for neurodegeneration.

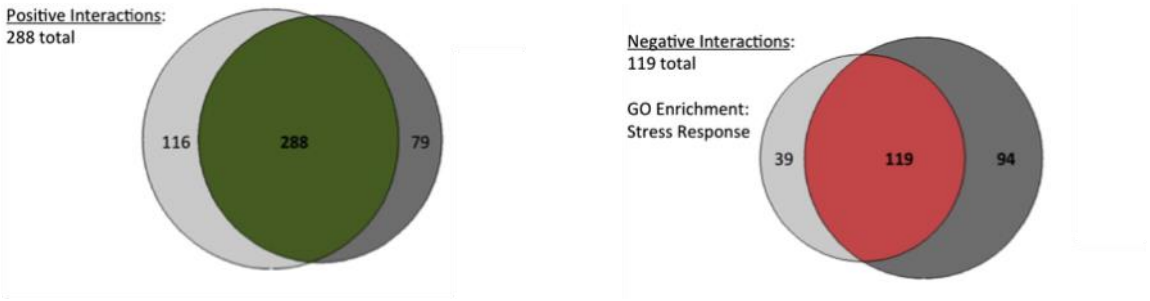


Fig. 3. The genetic interactions of btn1. SGAs were performed in two genetic backgrounds lacking btn1. This yielded 119 negative interactions (indicated in red), enriched for stress response, and 288 positive interactions (indicated in green).

Comparative gene expression analyses of 1-kb deletion models

Gene expression analysis in Cb*Cln3*$^{\Delta ex7/8}$ cerebellar precursor cells

The first milestone of this project part was a genome-wide microarray analysis of mouse cerebellar wild-type and *Cln3*$^{\Delta ex7/8}$ cell lines derived from a knock-in mouse model containing the most common *CLN3* mutation (1-kb deletion of exons 7 and 8) which is found in 85% of all CLN3 patients. In the time between application and start of the Dem-CHILD project, Dr. Cotman (Boston, MA), who had provided the project with these cell lines, already published a gene expression analysis of *Cln3*$^{\Delta ex7/8}$ cells (Cao et al. PloSone 2011), and found 981 dysregulated genes belonging to gene sets related to metabolic processes (fatty acid and amino acid metabolism and iron transport), protein transport or mitochondrial membranes. Therefore, it was decided to extent these studies by analyzing the expression of microRNAs in the same mouse cerebellar wild-type and *Cln3*$^{\Delta ex7/8}$ cell lines.

MicroRNAs are proposed as master regulators of gene expression in important cellular pathways. By analyzing their expression profile a deeper insight into the gene-regulating network in Cln3-defective brain supporting our studies on biomarker and modifier genes could be gained. To analyse microRNA data sets and to compare them with the already existing gene expression data sets from mouse models and patients, the advanced bioinformatic Ingenuity Software has been used. A concurrent license for this software had been purchased by the DEM-CHILD coordinator allowing its usage in a collaborative way by partners 1, 2 3, and 6 for WP04 as well as for WP 05.

A total of 21 up- and 8 down-regulated microRNAs have been identified in *Cln3*$^{\Delta ex7/8}$ cell lines. Up- and down-regulated microRNAs are supposed to act as suppressors and activators of the targeted mRNAs, respectively. From 19 of the 29 candidates, 663 mRNAs represent high potential transcripts estimated by filtering for metabolic processes, signaling, transport, and endocytosis. Among them, 117 mRNAs are experimentally demonstrated. For the remaining microRNAs no targets are known. Additionally, out of the 19 microRNAs we selected three microRNAs for further studies. In two of them, we could confirm the dysregulation by RT-PCR.

Also, the microRNA data were combined with the gene expression analysis data of the same CbCln3$^{\Delta ex7/8}$ cell line published by Cao et al. Approximately 500 dysregulated genes were found to be clustered and these included genes that may regulate potential biomarkers of CLN3 disease.

Secondly, an analysis of the total proteome of the *cln3 ki* cells was performed. For this purpose an *in vivo* protein labeling approach (SILAC; stable isotope labeling by amino acids in cell culture) followed by quantitative mass spectrometry (MS) was used. Approximately 200 dysregulated proteins were identified. Among these, candidates which correspond to potential targets of the previously identified microRNAs were found.

Taken together, this is the first comprehensive analysis of the lysosomal CLN3 proteome which identifies subsets of lysosomal enzymes impairing lysosomal homeostasis and lysosomal targeting routes that might represent novel targets for therapeutic approaches.

These data have been presented by Dr. G. Makrypidi at the 19th European Study Group on Lysosomal Disorders (ESGLD), Sept. 2013, in Graz, Austria, and at the Working Group for Paediatric Metabolic Society (APS) March 2014, in Fulda, Germany and a manuscript is being prepared:

Schmidtke C, Markrypidi G, Pohl S, Thelen M, Schweizer M, Jabs S, Storch S, Cotman SL, Gieselmann G, Braulke T, Schulz A: Cln3$^{Dex7/8}$ impairs endocytic receptor trafficking and efflux of lysosomal amino acids.

Evaluation of possible modifier genes on protein/mRNA level and in patient tissue

In parallel to the above described microRNA and pr0teome analyses, the gene expression data from mouse cerebellar *Cln3$^{\Delta ex7/8}$* cells were compared with genome-wide expression analyses in lymphocytes (PBMCs) of eight CLN3 patients homozygous for the 1 kb deletion who manifest different phenotypes characterized by fast, average and slow progression of the disease, and of six age- and gender-matched control individuals (Lebrun et al. Mol. Med 2011). In the latter study five genes were identified which were dysregulated in all CLN3 patients and present candidate biomarkers of the disease (Table 4).

RT-PCR analysis revealed that only DUSP2 (dual specificity phosphatase 2) is upregulated in all PBMCs collected form CLN3 patients of all phenotypes as suggested by the microarray data.

Further studies on DUSP2 have shown that the tissue and age-specific expression of DUSP2 appears to be a promising target to modulate both the onset and severity of CLN3 disease. A manuscript with detailed description of these studies is being prepared:

Makrypidi G, Schulz A, Ballabio A, Braulke T: TFEB control through ERK signaling by Dual Specificity Phosphatase 2.

Table 4: Biomarker candidates dysregulated in CLN3 patients (Lebrun et al. 2011)

		Fold change			
Gene	**Name**	**Slow**	**Average**	**Rapid**	**Confirmed by RT-PCR**
POLRJ2	DNA directed RNA polymerase II related	-2.6	-1.7	-2.2	-
CDC42SE2	CDC42 small effector 2	-2.5	-1.8	-3.2	-
RGS1	Regulator of G protein Signalling 1	+1.8	+2.5	+1.8	-
DUSP2	Dual specificity phosphatase 2	+2.4	+2.4	+2.4	+
PARP15	Poly(ADP-ribose) polymeraseFam. 15	+1.8	+1.7	+1.8	-

Comparative gene expression analyses in a model of acute down-regulation of CLN3

A genome-wide microarray analysis of acutely CLN3 down-regulated cells was performed. Acute down-regulation of *CLN3* by siRNA treatment in Hela cells represents a cell model for molecular alterations in CLN3 patients with severe loss of CLN3 function or rapid progression of juvenile CLN3 disease (Lebrun et al. Mol Med 2011). Thus, this cell model was used for the analysis of modifier genes in CLN3 disease. Treatment of HeLa cells for 96 hours with three different siRNAs resulted in a down-regulation of *CLN3* mRNA by 92 % in comparison with scrambled siRNA treated cells. The microarray analysis was performed in collaboration with SME partner 12. We identified in total 49 dys-regulated genes from which 7 were up-regulated and 42 were down-regulated. None of these genes were found within the gene data set derived from genome-wide microarray analysis derived from lymphocytes of CLN3 patients with rapid progression of the disease.

Conclusion

The work in WP4 has contributed to the identification of biomarker and modifier of CLN3 disease by

(1) identifying a novel signalling pathway and potential therapeutic targets, together with three small molecules in the yeast model

(2) comparative mRNA, microRNA arrays and SILAC protein analyses of cerebellar *Cln3$^{\Delta ex7/8}$* percursor cells providing new insights in the dysregulated gene network in CLN3 disease.

(3) evaluating the dual specificity phosphatase 2 (DUSP2) as a highly potential biomarker of CLN3 disease

WP05 Biomarkers and Modifiers of CLN1

Objectives

Utilise induced pluripotent stem (iPS) cells from patients and knockout mice to model the disease and to discover modifier genes for CLN1 disease

Utilise patient cell lines and cultured cell models to study the cell pathology caused by Mediterranean CLN1 mutations

Utilise MS based imaging (MALDI-IMS) in knockout mouse and in patient brain tissue for biomarker discovery

Development of a new microarray setup for the analysis of differential gene expression in NCL

Use of induced pluripotent stem (iPS) cells from patients and knockout mice to model the disease and to discover modifier genes for CLN1 disease

Utilization of established Cln1 miPS cell lines in production of neuronal cell cultures was unsuccessful and therefore the study was continued with CLN1 patient-derived hiPS cell lines. In order to get a more comprehensive picture of affected pathways and pathological changes in human CLN1 disease, iPSC and iPS-derived human neuronal cell models for CLN1 disease, classic infantile and CLN1 disease, late infantile representing three disease-associated mutations in four patients of Finnish or Italian origin were established and characterized.

To evaluate whether the generated CLN1 iPS cell lines and CLN1 iPSC-derived cell cultures have characteristic of NCL, we analysed the presence of autofluorescent storage material by immunofluorescence microscopy. While CLN1 hiPS cell lines were not found to accumulate storage material, these compartments were clearly visible in embryoid body-derived spontaneously differentiated cultures (Fig. 4). This indicates that generated CLN1 iPS cell lines have characteristics of the disease and therefore, could be utilised as a cell model in further functional analyses.

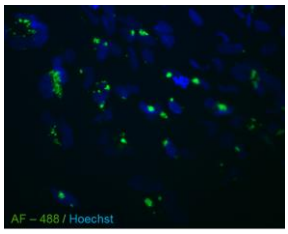


Fig. 4. Autofluorescent storage material in CLN1 hiPSC-derived spontaneously differentiated cell cultures visualised under 488 laser line. Representative example image of CLN1 hiPS cell line generated from CLN1, late infantile patient.

The utilisation of patient-specific iPSC cells and iPSC-derived cell cultures enables mapping of affected intracellular pathways in humans but additionally, searching for differences between phenotypically different patients. Therefore, control and CLN1 iPS cells, and iPSC-derived neurospheres and neurosphere-derived cultures were processed for whole transcriptome gene expression analyses by RNA sequencing. RNA sequencing analyses are currently in progress. Pathway analysis and verification of selected findings at protein level will be finalized post festum. These analyses may reveal further potential biomarkers for phenotypically different CLN1 disease subtypes.

Use of patient cell lines and cultured cell models to study the cell pathology caused by Mediterranean *CLN1* mutations

CLN1 patients' derived primary cell lines and cultured neuroblastoma cells have been used for the analysis of cellular biological defects in CLN1 disease. Transient transfection of SHSY5Y neuroblastoma cells with wild-type and a set of Mediterranean mutations in *CLN1* could be well achieved. This has been verified by testing with qPCR, and immunocyochemistry.

Modelled cells have been compared with primary cell lines to investigate:

i) autophagic processes both morphologically and molecularly with array techniques to detect variations in protein and mRNA expression;

ii) mitochondrial structure and function, namely the oxidative metabolism and ATP content, by combining immunocytochemistry and ultrastructure investigations with enzymatic assays

iii) oxidative and endolysosomal stress, induced by appropriate changes of growth conditions and use of specific chemicals, by qualitative and quantitative analysis of morphological and biochemical methods.

Results of these experiments have been confirmed by direct immunodetection at the protein level and might indicate sensible biological markers of the disease to be assessed in preclinical studies.

Results of this part of the project have been submitted for publication:

Scifo E. et al. Proteomic Analysis of the Palmitoyl Protein Thioesterase 1 Interactome in SH-SY5Y Human Neuroblastoma Cells.

Use of MS based imaging (MALDI-IMS) in knockout mouse and in patient brain tissue for biomarker discovery

MALDI-imaging mass spectrometry (MALDI-MSI) analyses on fresh-frozen mouse cortex tissues derived from 3(4) biological replicates at pre-symptomatic stage (1 month), symptomatic stage (3 months) and advanced stage (5 months), using 15-18 sections, 12 μm thick coronally cut sections from each brain has been completed. Proteomic profiling directly on tissues was performed by MALDI-MSI. Several dysregulated m/z in the range of 2-25 kDa were detected.

Laser Capture Microdissection (LCM) - based quantitative studies of the thalamus have been completed. Thalamic areas equal to ~ 500,000 μm^2 (+/- 3%) and corresponding to ~ 5000 cells, were coronally cut from each specimen at the same plane (from both genotypes and all age groups) to ensure maximum reproducibility. Extraction and trypsin digestion protocols were optimized to identify and quantify nearly 400 unique proteins in each LC-MS^E run. Label-free quantitation was performed in three technical replicates per sample at thresholds of $p< 0.05$, power >0.8 and fold change >1.2, that were used to determine a significant quantitative difference between the *Ppt1* $^{-/-}$ and wild-type age matched control thalamic tissues. Based on the above criteria, 35 proteins (7.6% of all quantified proteins) were significantly differentially expressed at the pre-symptomatic stage, 38 (9.6%) at the symptomatic and 81 (20.6%) at the advanced stage of CLN1 disease.

We linked the differentially expressed proteins in the *Ppt1*$^{-/-}$ thalamus with literature knowledge using Ingenuity Pathways (IPA) algorithms at the pre-symptomatic stage. In an effort to obtain a more comprehensive picture of the disease changes at this stage, we utilized Gene*MANIA* to extend the network analyses by bridging human orthologs of the differentially expressed mouse proteins with proteins in the same pathways/gene ontologies and PPT1.

Altogether, the proteomic data confirmed the earlier studies performed in *Cln1-/-* mice (von Schantz et al., 2008; Blom et al., 2013) as well as the first human CLN1 iPS cell analyses performed in DEM-CHILD WP5.

Results of this part of the project have been submitted for publication:

Tikka S et al. Proteomic profiling in CLN1 disease brain: an imaging and label-free proteomics approach.

Development of a new microarray setup for the analysis of differential gene expression in NCL

Control and CLN1 patient-derived hiPS cells, hiPSC-derived neurospheres, and neurosphere-derived cultures were processed for whole transcriptome analysis carried by RNA sequencing. RNA sequencing analyses have been performed.

Conclusion

Task 1: iPS cells from CLN1 patients having different CLN1 mutations, showed autofluorescence storage material typical to NCL. Live cell imaging of iPS cell derived neurons showed defective neuronal migration and a similar defect has been previously reported in Ppt1-/- mice (von Schantz et al, 2008). These data suggest that CLN1/PPT1 has a role in neuronal extension and migration. This defect was here observed for the first time in human NCL derived cells and represents one of the earliest pathological changes in the CLN1 disease.

Task 2: Cultured neuroblastoma cell models overexpressing mutant CLN1 as well as CLN1 patient fibroblasts shoed enhanced apoptotic cell death after Staurosporin (STS) induction. Hyperpolarization of mitochondrial membrane was observed in both cell populations and CLN1 deficient cells showed dramatic shift to depolarized stage following STS treatment. Immunolabeling of CLN1 patient fibroblasts confirmed fragmented mitochondrial reticulum. Also, decrease of ATP production and reduction of mitochondrial mass was observed. Altogether, patient derived CLN1 fibroblasts showed a major defect in mitochondrial function.

Task 3: Proteomic profiling of Ppt1-/- mouse brains using MALDI-MS at presymptomatic stage thalamus revealed several changes related mostly to metabolic processes. In the symptomatic stage the changes were additionaly related to mitochondrial dysfunction, synaptosomal vesicle trafficking and signalling cascades, including RhoA signaling involved in neuronal extension and pathfinding. In the advanced stage, also strong dysregulation of myelin proteome was confirmed. Altogether, these data performed with MALDI-MS proteomics confirmed the earlier studies performed in Cln1-/- mice (von Schantz et al., 2008; Blom et al., 2013) and human CLN1 iPS cell analyses in this WP.

WP06 – Innovative therapies for NCLs caused by mutations in transmembrane proteins

Objectives

To explore the challenges that will face the development of therapies for NCL disease caused by mutations in intracellular transmembrane proteins (specifically juvenile CLN3 disease and CLN6 disease)

To determine whether any of the following approaches provide benefit

(1) over-expression of CLN3 and CLN6 is toxic to cells

(2) AAV-mediated expression of CLN6 delays retinal or brain neurodegeneration

(3) neural stem cell-mediated intraocular delivery of neuroprotective factors provides benefit.

Production of AAV vectors

AAV constructs carrying the human or murine *CLN3* gene under control of the CMV promoter were cloned. The constructs also contained a GFP marker gene (IRES-driven) that allowed the identification of transduced cells. The CLN3 constructs were packaged into AAV9 capsids, as this vector serotype was expected to be effective in transducing neurons after administration into the central nervous system. Similar AAV constructs carrying the human or murine *CLN6* gene were cloned and packaged into the AAV8 serotype, a serotype known to transduce photoreceptor cells after subretinal delivery into the eye. Control vectors carrying the GFP marker gene (AAV-GFP) or without a transgene (AAV-empty) were produced for both serotypes to control for effects relating to vector administration. All vectors were assessed for quality (endotoxin levels, non-AAV proteins) and titres. Where possible, the expression of transgene was assessed *in vitro* prior to use of the vectors *in vivo*.

Production of genetically engineered neural stem cell cultures with forced expression of one or more neuroprotective factors

To evaluate whether a sustained cell-based intraocular administration of neurotrophic factors (NTF) results in attenuation of photoreceptor degeneration in in a CLN6 mouse model, different neuroprotective NTFs (glial cell line-derived growth factor (GDNF), brain-derived growth factor (BDNF), pigment epithelium-derived growth factor (PEDF), ciliary neurotrophic factor (CNTF)) were expressed in neural stem (NS) cells derived from the cerebral cortex of embryonic mice. When these cells are cultivated under adherent conditions in the presence of mitogens, they give rise to homogeneous cultures of symmetrically dividing tripotent neural stem (NS) cells. To modify these cells, the cDNAs of the different NTFs were cloned into polycistronic lentiviral vectors additionally encoding different combinations of reporter and resistance genes under regulatory control of the cytomegalovirus enhancer/chicken ß-actin (CAG) promoter. NS cells for control experiments were modified with the same vectors but lacking the cDNAs for the NTFs. Because expression levels of the NTFs from the polycistronic vectors are proportional to the expression levels of the fluorescent reporter proteins, single NS cells with the highest expression levels of the reporter genes from transduced bulk cultures were selected by fluorescence activated cell sorting (FACS), and clonally expanded to generate clonal cell lines with elevated expression levels of NTFs. After several rounds of transductions and clonal expansions, several BDNF-, GDNF-, PEDF- and CNTF-expressing NS cell lines were selected with different expression levels of the transgenes for further characterization and for transplantation experiments. Immunocytochemical and Western blot analyses revealed stable expression of the NTFs in these cell lines, and confirmed secretion of the factors into the culture supernatants. When the modified cells were differentiated into astrocytes or neurons in vitro, they continued to express the NTFs. Stable expression of the transgenes was also observed in intravitreally transplanted cells over a time period of at least six weeks, the longest post-transplantation interval analyzed.

Detail onset and progression of retinal pathology and visual defects in CLN6 & CLN3 mouse models

To allow treatment efficacy to be assessed, survival of photoreceptor and retinal neuron subclasses, glial activation, integrity of retinal pigment epithelium, accumulation of autofluorescent storage material, optic nerve pathology, and extent of remaining visual function have been assessed by optomotor testing and electrophysiological measures of visual function (ERGs and VEPs) in different ages of C57Bl6/J congenic CLN6 and CLN3 mouse models. These findings have been correlated with existing knowledge on the integrity of the visual system in these mice and provide landmarks of disease progression that are crucial for assessing the impact of therapy in the visual system.

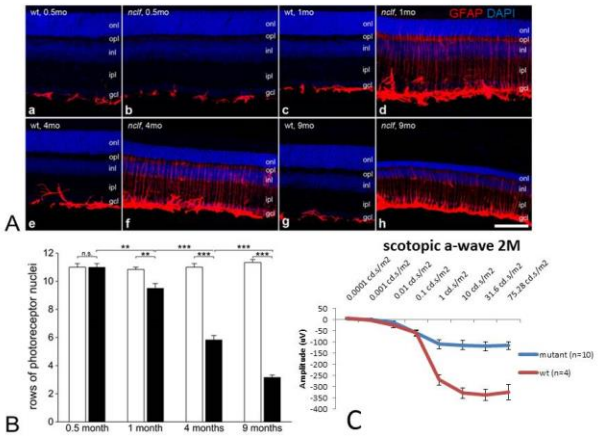
CLN3 mouse model

Assessment of the retina in the CLN3 mouse revealed that there was no consistent retinal pathology or visual defect in this animal model within the first year of life. As the initial administration of a therapeutic is generally associated with a small acute drop in visual function/retinal thickness (especially after subretinal administration), the very slowly progressing loss of photoreceptors in the CLN3 mouse created an unsurmountable hurdle to the development of a novel therapeutic regimen. For this reason, these mice have not been used in the development of ocular therapies.

CLN6 mouse model

Detailed characterisation of the ocular phenotype in the mouse model of vLINCL (*Cln6^{nclf}* mouse) allowed to create a morphological and functional description of the disease phenotype. Analyses of retinas from *Cln6^{nclf}* mice at different developmental ages revealed a slowly progressing photoreceptor degeneration. Apoptotic photoreceptors were present at 2 weeks of age, with loss of photoreceptor cells evident at the end of the first postnatal month, and nearly complete in 9 months old mice (Fig. 5 A). Retinal degeneration (Fig. 5 B) was paralleled by elevated expression levels of glial fibrillary acidic protein in astrocytes and Müller cells (Fig. 5 A), and a dysregulation of lysosomal proteins such as cathepsin D and Lamp1 as revealed by immunohistochemistry, Western blot analyses and enzyme activity assays. Furthermore, electron microscopic analyses revealed the presence of lysosomal storage material with curvilinear and fingerprint-like inclusions in various retinal cell types. Characterisation of retinal function at various ages, identified early changes to the a- and b-wave form in electroretinography. A decrease in scotopic (dark-adapted) a-wave amplitude (a function of the rod photoreceptors) was present as early as 3 weeks of age, the youngest age at which reliable ERG measurements can be performed. The loss of function progressed rapidly before slowing around 3 months (Fig. 5 C). The scotopic b-wave (a measure of rod bipolar cell function) was significantly lower than in WT mice, but this difference was not progressive. No notable differences were present in the photopic (light-adapted) ERG. The morphological and functional characterisation of the animal model suggests that the rod photoreceptor cells are the primary cell type affected in this disease.

To conclude, the defined steps of photoreceptor degeneration suggest that nclf mice might serve as an ideal animal model for experimental therapeutic approaches aimed at attenuating vision loss in neuronal ceroid lipofuscinosis. *These results have been published by Bartsch U et al. Invest Ophthalmol Vis Sci (2013).*



Bartsch U et al. 2013

Fig. 5 Disease phenotype in retinas of Cln6^{nclf} mice. (A) The photoreceptor layer (onl) was similar in thickness in 0.5 months old wild-type (a) and Cln6^{nclf} mice (b), and expression of GFAP was restricted to retinal astrocytes in both genotypes (a, b). In 1 month old mutants (d), the photoreceptor layer was slightly reduced in thickness when compared to age-matched wild-type mice (c). Photoreceptor loss was more pronounced in 4 months old mutants (f), and almost complete in 9 months old Cln6^{nclf} mice (h). Retinal degeneration in CLN6^{nclf} mice was paralleled by elevated expression levels of GFAP in retinal astrocytes and Müller cells (d, f, h) when compared to age-matched wild-type mice (c, e, g). (B) The number of rows of photoreceptor nuclei was similar in 0.5 month old CLN6^{nclf} and wild-type mice, but then decreased in Cln6^{nclf} mutants with increasing age of the animals. In 9 months old mutants, the photoreceptor layer was composed of about 3 rows of photoreceptor nuclei as opposed to about 11 rows of nuclei in age-matched wild-type retinas. (C) Scotopic a-wave amplitude, a measure of rod photoreceptor activity, was affected in 1 month old Cln6^{nclf} mice, decreasing rapidly over the subsequent months (data shown at 2 months of age)

AAV-mediated gene transfer in CLN6 & CLN3 mouse mutants

Retinal gene therapy in *Cln6^{nclf}* mice

The characterization of the visual phenotype revealed that *Cln6^{nclf}* mice present with photoreceptor cell death and reduced photoreceptor activity from 2 weeks and 3 weeks of age, respectively. These findings indicate firstly that the mutation in *Cln6* predominantly affects photoreceptor survival and secondly that therapeutic interventions most likely need to be administered before the age of 2 weeks to prevent the onset of the retinal degeneration.

To assess toxicity associated with transgene expression, the first step in the development of gene therapy, AAV2/8.cmv.hCLN6 vectors were produced as described above and injected subretinally into eyes of adult wild type animals. A range of titers of AAV2/8 vectors carrying *Cln6* has been used.

To conclude, preliminary data showed that high expression levels of Cln6 are detrimental to retinal function and morphology. Currently, lower expression levels of Cln6 in photoreceptor cells are being assessed for their therapeutic efficacy.

Kleine-Holthaus et al., presentation at the 14th International Congress on Neuronal Ceroid Lipofsucinoses, Oct. 2014, Cordoba, Argentina and will be published soon.

Delivering gene therapy to the brain in Cln3-deficient mice

Because the cross-correction of adjacent cells is not possible in disorders caused by a transmembrane protein deficiency, the requirements of a gene therapy vector may differ to those needed in an enzyme deficient form of NCL. Since overexpression of *Cln3* is associated with cell toxicity in *Drosophila*, we also needed to characterise whether vector driven Cln3 expression has similar effects, or is associated with localised inflammation. We have been assessing the capacity of an AAV2/9-CMV vector to drive either the reporter gene GFP alone (AAV2/9-CMV-GFP), or a bicistronic vector expressing both GFP and either mouse Cln3 (AAV2/9-CMV-GFP-mCln3) or human CLN3 (AAV2/9-CMV-GFP-hCLN3). This was addressed first *in vitro* in primary cultures and subsequently *in vivo*, to determine the cell-type specificity of transduction and whether adverse events are associated with gene delivery. These studies revealed that a high multiplicity of infection was needed to produce even low-level transduction of cells *in vitro*, that neurons were transduced with higher efficiency than glia, and that bicistronic vectors appeared to express GFP at lower levels. Most importantly, at these titres none of these vectors resulted in any overt cytotoxicity.

We next assessed whether these AAV2/9 vectors were capable of similar transduction at these titres *in vivo.* This analysis revealed widespread transduction, and an acute astrocytosis and microglial activation that was mostly localised to near the injection tract, but declined with increased survival time. Based on these data we designed a larger study to determine the extent of vector transduction, inflammatory response and cytotoxicity in wildtype and Cln3 deficient mice at two different ages. These mice were injected unilaterally (with 1μl at 1 x 10^{12} titre) into the striatum at either 3 months (presymptomatic) and 7.5 months of age (when pathological changes are already evident in these mutant mice), with the contralateral uninjected hemisphere serving as a within-animal control. In order to control for the effects of inserting a needle into the brain, and for injecting a volume of fluid into the striatum, additional sham injected (needle inserted, but no injection made) and vehicle injected mice were also generated. All mice were examined histologically at either 2 and 6 months post injection, with a smaller number of mice analysed by quantitative PCR to determine transgene expression. These cohorts of mice have all been sacrificed and the histological analysis is nearing completion.

To conclude, preliminary data suggest that AAV2/9 vectors appear to be capable of widespread and long lasting transduction of the CNS..

Intraocular transplantation of neural stem cells engineered to (co-)express neuroprotective factors

To evaluate the clonal cell lines for potential neuroprotective effects on photoreceptor cells, they were intravitreally grafted into CLN6^{nclf} mice at the onset of retinal degeneration at postnatal day 14. The contralateral eye of each animal received injections of control NS cells and served as a reference. Photoreceptor numbers in NTF-treated and control eyes were determined 2, 4 and 6 weeks after transplantation in six defined regions of the nasal and temporal retina (Fig. 6). Several different NTF-secreting cell lines (i.e. BDNF-, GDNF-, PEDF- and CNTF-secreting NS cell clones) and combinations of BDNF-, GDNF- and PEDF- expressing NS cell lines have been analysed for the effect on retinal degeneration. Only grafted CNTF-expressing NS cells were capable to attenuate the loss of photoreceptor

cells in CLN6^{nclf} mice. These results encourage further research into neuroprotective approaches aimed at attenuating retinal degeneration in NCL disease caused by mutations in intracellular transmembrane protein.

Results of this project task have been submitted for publication: Jankowiak W et al. Sustained neural stem cell-based intraocular delivery of CNTF attenuates photoreceptor loss in the nclf mouse model of neuronal ceroid lipofuscinosis.

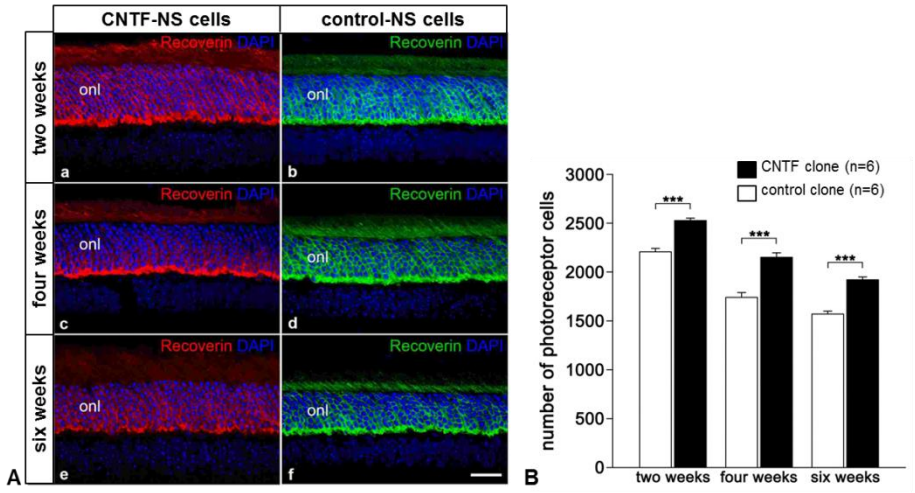


Fig. 6: Intravitreally grafted CNTF expressing neural stem cells attenuate photoreceptor degeneration in nclf mice.(A) A CNTF expressing NS cell line was intravitreally grafted into 14 days old nclf mice, and retinas were analyzed two (a), four (c) and six (e) weeks after transplantation. The contralateral eye of each animal received injections of control NS cells and served as a reference (b, d, f). A comparison of central retinal sections revealed a significantly thicker photoreceptor layer (onl) in CNTF-treated retinas when compared to the contralateral control retinas. (B) Determination of photoreceptor numbers in six defined areas of the central nasal and temporal retina revealed the presence of significantly more photoreceptor cells in CNTF-treated retinas (filled bars) than in control retinas (open bars) at all post-transplantation intervals analyzed. Each bar represents the mean value (±SEM) from six retinas. ***, p<0.001 (Newman-Keuls post hoc test after the mixed two-way ANOVA)

Conclusion

The **retinal *CLN6* gene therapy study** has shown that gene supplementation of the *CLN6* gene to the mouse photoreceptor cells is insufficient to attenuate photoreceptor cell degeneration. Future experiments will aim to target the bipolar cells specifically.

The ***CLN3* gene therapy study** analysed the delivery of CLN3 to the CNS showing that AAV2/9 vectors are capable of widespread and long lasting transduction of the CNS.

The **study using genetically modified neural stem cell grafts to deliver neurotrophic factors to the retina** has shown that intravitreally grafted CNTF-secreting stem cell lines are capable of attenuating the loss of photoreceptor cells in CLN6^{nclf} mice. These results encourage further research into neuroprotective approaches aimed at attenuating retinal degeneration in NCL disease caused by mutations in intracellular transmembrane protein.

WP 07 – Identification of new NCL genes

Objectives

To identify novel NCL genes by

1. collecting new families, especially those that are consanguineous or multiply affected, focusing on those newly diagnosed from India
2. excluding known gene loci from new families
3. performing exome sequencing of excluded families as means of gene identification
4. confirming / excluding candidate variants by segregation analysis and by screening appropriate control individual
5. analysing the newly identified genes for mutations in known but genetically undefined NCL families

Collection of families with genetically undefined NCL

This task was extended to include genetically undiagnosed families received by any route, to supplement those received from India diagnosed with NCL (n=45). In addition to those families submitted by partners of DEM-CHILD, we received some Indian families through independent contact of participants by Indian clinicians. We also collected families through other European and international contacts. A total of 363 families were received requiring diagnosis from countries of the DEM-CHILD consortium plus 10 additional countries.

Excluding known gene loci from new families

Patient numbers diagnosed from DEM-CHILD countries are reflected in the epidemiology studies in WP03. In addition, 31 patients from countries outside the consortium were also found to carry mutations in known genes (*CLN1, CLN2, CLN5, CLN6, CLN7, CLN8, CLN11*). Mutation analysis of known genes has been performed in those families confirmed with NCL. Data on known and new mutations identified have been added into the NCL mutation database curated by partner 1. More are likely to be added beyond the end of the project.

About half the mutations were found in the gene *CLN2*, and 17% in both *CLN6* and *CLN7*, suggesting that about 75% of new undiagnosed cases that have been excluded from *CLN1* and *CLN2* loci by rapid enzyme testing may represent CLN6 and CLN7 diseases. Interestingly no cases were found to carry mutations in *CLN3,* suggesting that recognition of classic juvenile CLN3 disease, is improving in these countries or that few cases are present.

Identification of new loci using exome sequencing

A total of 209 families were submitted for analysis by targeted sequencing to partner 6 with many positive hits identified, and nine families were submitted for independent analysis by partner 3.

Results of the deep NGS screens are still under inspection between partners, and there are indications for a number of yet unknown mutations. E.g., in one of the samples from India there is a deletion of 3 codons in the *PCLO* gene, which is one of the NCL candidate genes. The deletion could change the conformation of the encoded protein and seems to be homozygous. PCLO (homo sapiens piccolo presynaptic cytomatrix protein) is part of the presynaptic cytoskeletal matrix, which is involved in establishing active synaptic zones and in synaptic vesicle trafficking. Variations in this gene have been associated with bipolar disorder and major depressive disorder

Activities under this task on families collected from India have been negatively affected by the restriction of the Indian partner to provide samples to the project. However, some other locally held families have been subject to exome sequencing, through local collaboration. In families with childhood or teenage onset, this has resulted in the identification of one new disease gene ATP13A2/CLN12 in a Belgian family (Bras et al. 2012). All four affected siblings showed difficulties in learning from around the ages of 8 years characteristic for dementia, followed by rigidity, and akinesia. Both, vacuolated lymphocytes and ultrastructural pathology that resembled that for NCL were diagnosed but no mutation underlying the disease had been identified. Exome sequencing led to the identification of a single homozygous mutation in *ATP13A2* Mutations in ATP13A2 are a known cause of Kufor-Rakeb syndrome (KRS), a rare parkinsonian phenotype with juvenile onset. These data show that NCL and KRS may share etiological features and implicate the lysosomal pathway in Parkinson's disease.

Conclusion

On new gene, ***ATP13A2*** has successfully been identified in a collaborative project of partner 3.

WP08 Teaching and Dissemination

Objectives

Teaching and training with regard to diagnosis and treatment of NCL disorders addressing

(a) experts in NCL diseases – to maintain best clinical practice

(b) non-expert physicians - to increase awareness of these diseases.

(c) other medical professionals (lab technicians, nurses and other health care providers)

(d) parents, family members, and caregivers of patients

Dissemination of scientific results obtained during the project

Dissemination of diagnostic algorithm for NCL disorders

Since the DEM-CHILD grant application in 2011, the number of known NCL genes has increased from eight to at least thirteen (Warrier V et al. BBA 2013). Thus, the already existing agreed-upon diagnostic algorithm had to be changed accordingly and updated. A DEM-CHILD diagnostic algorithm for NCL diseases had been developed in collaboration with all NCL clinical experts in the consortium and published as part of a review on clinical presentation of NCL diseases (Schulz A. et al, BBA 2013).

Workshops

Six DEM-CHILD teaching workshops focussing on this diagnostic algorithm have been organised during the entire project period (Table 5 and below).

Handouts to medical professionals

A flyer illustrating both, this diagnostic algorithm for NCL diseases as well as providing information where to send diagnostic samples, has been designed and distributed not only during the respective DEM-CHILD teaching seminaries but also at various international congresses (see list of dissemination activities). The flyer can also be downloaded for free from the DEM-CHILD website.

Project website

A project website has been created and can be accessed under http://www.dem-child.eu. The website provides information regarding the new diagnostic algorithm (handout can be downloaded for free), as well as information regarding addresses, contact persons, institutions to where and under which conditions diagnostic samples should be sent in the specific country.

Teaching video

A teaching video on NCL has been created with the support of the German NCL Family Association. For this purpose, German NCL patients gave permission to be recorded during neurologic exams, EEG, MRI etc. when they visited the NCL clinic at the University Medical Center Hamburg-Eppendorf, Germany (partner 01). The video has already been given to NCL families at the Annual Meeting of the German NCL Parents Association in September 2012. Once translated, it will be distributed during the project's next diagnostic workshops (see above) and can also be ordered for free via the project's website.

Teaching of performance and evaluation of diagnostic procedures

As mentioned above, a total of six DEM-CHILD teaching workshops have been organised during entire project. In order to keep travel costs low for participants, most workshops have been organised before /after or as part of international conference (Table 5).

During these workshops, not only the diagnostic algorithm but also performance and evaluation of diagnostic procedures such as electroretinogram (ERG), electroencephalogram (EEG), light microscopy of blood smears (LM), electron microscopy (EM), and cMRI have been taught by the respective specialists. Each workshop has been

attended by approximately 80-100 participants such as paediatricians, child neurologists, adult neurologists, care givers, lab technicians etc.

Table 5: List of DEM-CHILD Teaching Workshops during project period

	Conference	Date	Location
1	13th International Conference on Neuronal Ceroid Lipofuscinoses	31st March 2012	London, UK
2	DEM-CHILD General Assembly Meeting	19th Oct 2012	Verona, Italy
3	Annual Conference of the German, Swiss and Austrian Child Neurology Society	25th April 2013	Innsbruck, Austria
4	10th European Pediatric Neurology Society Meeting	25th Sept 2013	Brussels, Belgium
5	Annual Meeting of the Association of Child Neurology India	6th Feb 2014	Hyderabad, India
6	International Workshop on Affinity-Mass-Spectrometry in Life Sciences	24th Nov 2014	Rüsselsheim, Germany

Dissemination of current research in NCL

Dedicated to consortium

Dissemination of current results of the different work packages of the consortium was performed during 6-monthly Steering Committee and annual General Assembly Meetings (refer to WP09). During these meetings, each WP leader gave a detailed presentation of the current research in the respective work package. Moreover, during General Assembly meetings, young scientists gave presentations of their projects and discussed them with the consortium. Each WP presentation from these meetings is available at :milliarium, a web based tool for FP7 project management provided by GABO:mi. By using :millarium, members of the consortium can access all WP presentations, access the minutes summarizing main results from each DEM-CHILD meeting and update their milestone list.

Dedicated to interested groups outside the consortium

Members of the consortium have given several talks and presented posters about the DEM-CHILD project to NCL experts as well as non-expert child neurologists, geneticists, other clinicians and scientists across the world in various cities including those in the UK, Italy, Germany, India, Finland, Ireland, and USA. Over 2000 international professionals have participated in these conferences. One recent dissemination highlight has been the 14th International Conference on Neuronal Ceroid Lipofuscinoses and Patient Organisation Meeting in Cordoba, Argentina, in October 2014. During this international meeting, members of the DEM-CHILD consortium have disseminated results from their respective work package in a total of ten talks and 5 poster presentations. For a detailed list of DEM-CHILD posters and presentation during scientific meetings, please refer to the dissemination list in ECAS.

Dedicated to the general public

NCL family associations

Throughout the entire project period, the DEM-CHILD consortium has closely collaborated with the national NCL family associations in the participating countries: Clinical experts have given talks at the annual meeting of the Italian, German, Finnish and UK family associations meetings. Moreover, they have provided written reports on the DEM-CHILD work to the NCL family community to be published yearly in the national family associations' information journal which is sent out to all NCL families in the respective country.

General public

Several interviews with public newspapers as well as during TV-shows have been performed by various DEM-CHILD partners. For a detailed list of DEM-CHILD dissemination activities to the general public, please refer to the dissemination list in ECAS.

Conclusion

In **WP8**, a NCL diagnostic algorithm has been developed, published and disseminated during various DEM-CHILD teaching seminars including in India. Results of current research of the project have been distributed via workshops, conferences, publications and articles. The dissemination activities reached different target groups such as clinicians, scientists, and patient's families.

WP09 Project Management

Objectives

To ensure the proper scientific management of the project, to strengthen and support the Participants to achieve the objectives, and complete milestones and deliverables in time

To make sure that the consortium's contractual duties are carried out. This includes not only the compliance with EU regulations and their contractual, financial and legal requirements towards the Commission, but also within the Consortium (Consortium Agreement)

To set-up an effective communication infrastructure within the consortium

To manage the capture and protection of intellectual property, including confidential information and patents

To ensure that produced foreground is disseminated to the relevant target groups through publications and training

Consortium management tasks and their achievement

The management of DEM-CHILD was the main task of the scientific and administrational Project Office, i.e. the coordinator and his administrative assistant at UKE and GABO:mi. It includes the following:

- Ensure efficient functioning of the project in order to i) achieve the objectives, ii) complete the milestones in time and iii) to deliver the deliverables.
- Make sure that the infrastructure's contractual duties are carried out. Support and strengthen the participants to comply with the EU regulations and their contractual and legal requirements. Abide by the "good practice" of resources management as presented in the Financial Guidelines.
- Set-up an effective communication infrastructure and foster the integrative process within the consortium.
- Manage the processes for the capture and protection of intellectual property, including confidential information, copyright, design rights and patents properly.
- Ensure that produced foreground is disseminated to the relevant target groups through publications and training in close collaboration with WP 8 Dissemination and Training

Management of legal issues:

A consortium agreement was established, discussed with partners and their legal departments, agreed and signed by all partners' legal departments. All signatures are available at the project office.

One contract amendment was done (approval on 13th Feb 2013). The reasons for this amendment were:

- the addition of participant 11, Costal Research and Management GbR (CRM),
- a special material transfer agreement with the Indian Council of Medical Research and the modification of Annex I.

A second contract amendment was done (approval on 31st Oct. 2014). The reasons for this amendment were

- the accession of beneficiary 12 Source Bioscience plc. Due to structural changes at Benficiary 06 imaGenes GmbH and latest developments in sequencing technologies, certain subtasks of WP1 need to be fulfilled by the mother of imaGenes GmbH: Source Bioscience plc.
- the cancellation of the incidence study and corrective action of a targeted population screening of 500 dry plod spots, collected in selected patient groups
- in combination with targeted population screen the accession of beneficiary 13 Steinbeis GmbH to the consortium to have the possibility to develop a MS-based multiplex array for CLN1, CLN2 and CLN10 for the improvement the automated enzyme testing.
- the possibility to invite participants of the DEM-CHILD Data Base Consortium to attend the training day in September 2014.

Communication Management:

- GABO:mi ran the organisational and administrative part of the project office and was available as a permanent helpdesk for all participants
- Key aspects were communicated to or discussed with the Scientific Officer of the European Commission
- Phone conferences with the Coordinator and relevant partners were carried out to discuss actual progress within the project.
- All relevant information are available on :milliarium (web-based tool for FP7 project management) and can be presented upon request.
- GABO:mi served as an helpdesk for questions which arose at partners of the DEM-CHILD consortium.

Organisation and Coordination of the work programme

DEM-CHILD consisted of 9 work packages (WPs). Each WP was carried out by a varying number of involved parties. The WP leader supervised and adjusted the process flow and worked closely with the project office. He or she distributed tasks to the WP members and was held liable for the overall success of the WP. Reports on the status of the WP have been given to the Steering Committee at regular intervals. WP meetings and phone conferences have been arranged as necessary. The activities of the WP have been overseen by the chairs of each working group, the WP leaders.

Project Controlling:

Part of the quality control was a professional Project Controlling, including the following issues:

- To oversee the compliance of the project with the EU provisions (grant agreement and its annexes) and the Consortium Agreement
- To prepare templates concerning the extent, structuring and the description depth of information on the status of the project and the progress
- To manage the due-process submission of reports (according to the reporting guidelines for FP7)
- To coordinate the submission of deliverables and milestones particularly with the WP leaders
- To monitor and regularly present the status and progress with :milliarium to the coordinator and the boards.

Finance Management:

The Coordinator and GABO:mi were responsible for the central finance controlling. GABO:mi has long experience in financial processing of government-supported collaborative research projects and has developed :milliarium especially for the error-free finance controlling.

Activities were as follows:

- Coordinate and transfer the EC payments to the participants following the approval of the first periodic report Monitor costs vs. budgets on an annual basis
- Compile and discuss several budget shifts for Amendment N°2
- Report on financial issues as required by the reporting guidelines
- Support all partners with issuing Form Cs and obtaining audit certificates in due time
- Close cooperation with the administration departments of the involved parties if necessary
- Coordination and monitoring of payments to the project parties

Meeting Organisation:

Organisation of effective meetings of the Consortium

- Yearly meetings of the General Assembly
- Half-yearly meetings of the Steering Committee

Organisation comprises the following tasks:

- Professional convocation and on time shipping of the agenda according to the terms of the Consortium Agreement
- Organisation of conference venues with the required infrastructure and catering, ensure a smooth running of the conference
- Support with the meeting preparation for the WP leaders (focus of presentations, templates, structuring)
- Organisation and steering of decision-making processes at DEM-CHILD meetings
- Follow-up of points agreed at the meetings, therefore writing and circulation of SMART minutes (Specific, Measurable, Agreed, Results-oriented, Time framed) and monitoring of pursuance.

Type of meeting	**Date**	**Venue**
Kick off Meeting (1st General Assembly, 1st Steering Committee Meeting)	1 – 3 October 2011	Hamburg, Germany
2nd Steering Committee Meeting	1 April 2012	London, United Kingdom
2nd General Assembly and 3rd Steering Committee Meeting	18 – 20 October 2012	Verona, Italy
4th Steering Committee Meeting	7 March 2013	Munich, Germany
3rd General Assembly Meeting 5th Steering Committee Meeting	2 - 4 September 2013	Helsinki, Finland
6th Steering Committee Meeting	22 January 2014	Frankfurt, Germany
4th General Assembly Meeting 7th Steering Committee Meeting	1 – 3 September 2014	Hamburg, Germany

Project Website

The project website has been updated regularly according to latest results of the project. It also gives an overview on the publications in the frame of DEM-CHILD. The DEM-CHILD website will continue to stay online and will especially focus on the continued and future long-term work of the DEM-CHILD NCL patient database consortium.

The password protected project management tool :milliarium has been used as platform to dissemination of current results among all consortium members including young investigators. It was updated regularly, at least every 6 months. Meeting presentations are available for download as well as the project flyers, manuscripts and hand out material for further dissemination activities.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

Improving Health of European citizens

The improvement of health of European citizens as well as the increase of competitiveness of innovative capacities of European health-related industries and businesses are the key aims of the European Health theme. The DEM-CHILD project has a significant impact on key EU social and economic issues by increasing and reinforcing European Brain Research and its competitiveness in this domain. Moreover, the DEM-CHILD project clearly addresses the main goals of the International Rare Disease Research Consortium (IRDiRC) to team up scientists from academia and industry and organisations investing in rare disease research in order to achieve two main objectives by the year 2020, namely 200 new therapies for rare diseases and means to diagnose most rare diseases. In addition, the DEM-CHILD project addresses the recommendations of the Consensus document on European brain research issued by the European Brain Council (2006), both in its design (multidisciplinary effort of scientists from academia and industry together with clinicians) and its expected impacts: improved understanding of molecular mechanisms of the neurodegenerative diseases, innovative options to treat or prevent these disorders and increased innovation and competitiveness of European brain research.

The DEM-CHILD project has focused on a group of neurodegenerative diseases, the neuronal ceroid lipofuscinoses (NCLs). As the most frequent cause for childhood dementia in Europe, they represent an important problem for society. Even though the NCLs fall under the definition of “orphan diseases”, they are nevertheless the most common neurodegenerative diseases of childhood leading to dementia, blindness, motor deterioration and epilepsy. To date, none of the ten different NCL diseases can be cured, and as such they all lead to an early death of the patients, usually after a protracted period of disability. In this respect these diseases place a burden of long-term suffering upon the affected patients and their families, as well as a major socio-economic burden upon health care systems.

In order to advance the development of treatment options for the NCL diseases, the DEM-CHILD project has increased knowledge of the biological processes and mechanisms involved in the NCL diseases, especially CLN3 disease (WP4) and CLN1 disease (WP5) and their impact on the clinical spectrum of these diseases. This knowledge has been successfully transferred into clinical applications for prevention and early detection of these diseases (WP1, WP2, WP3), and ensuring that clinical (including epidemiological) data guide further research towards the establishment and evaluation of innovative therapies (WP3).

The new therapeutic options for NCL patients arising from the DEM-CHILD project (WP6) have the potential to make a substantial impact on improving the health of children suffering from childhood dementia, retinal and neurodegeneration in Europe. In addition, DEM-CHILD research has a significant effect on the understanding of biological processes leading to visual and brain dysfunction and the establishment of innovative therapies of neurodegenerative diseases in general, including age-related diseases, e.g. Alzheimer disease, which affect an even larger proportion of the European population.

Methods for health promotion and prevention including promotion of child health

Prevention of paediatric neurodegenerative diseases in Europe and India

With one exception, all NCLs are inherited in an autosomal recessive fashion, which means that there is a significant risk of multiple children being affected in one family. For most families there will be no prior clue that both unaffected parents carry an NCL disease-causing mutation. Moreover, affected children appear to be completely healthy during the first years of life. So by the time an affected child shows the first clinical symptoms and the right diagnosis is finally made, younger brothers and sisters might have already been born, each carrying a 25% risk of also being affected by this NCL disease. As such, a family may find that several or all children are affected by an incurable neurodegenerative disease, leading to immense suffering and the early death of these children.

However, the sooner a genetic diagnosis of NCL is made in a family, the lower is the risk of multiple children being affected, because the family can be provided with genetic counseling and the opportunity of prenatal testing for NCL to ensure delivery of an unaffected child. In addition, relatives can be tested for being carriers and also obtain genetic counseling as well.

DEM-CHILD project has aimed at preventing the risk of multiple affected children with NCL in one family, and at reducing the risk of occurrence of NCL disease in general by reducing the time and associated costs for making a genetic diagnosis. Multiple actions have been taken to achieve this aim:

(1) Increase awareness of NCLs

(2) Development and dissemination of a new diagnostic algorithm for NCLs

(3) Development of new testing and screening methods applicable to the wider community

(1) Increase awareness of NCLs

As described in WP08, members of the DEM-CHILD consortium have given numerous talks and presented posters about NCLs in general and the DEM-CHILD project in particular to NCL experts as well as non-expert child neurologists, geneticists, other clinicians and scientists across the world in various cities including those in the UK, Italy, Germany, India, Finland, Ireland, and USA. Over 2000 international professionals have participated in these conferences. These efforts have significantly increased awareness of NCLs in the scientific and also medical community.

In order to increase awareness for the general public, several interviews with public newspapers as well as during TV-shows have been performed by various DEM-CHILD partners. For a detailed list of DEM-CHILD dissemination activities to the general public, please refer to the dissemination list in ECAS.

A total of six DEM-CHILD teaching workshops have been organised during entire project, specifically one in Hyderabad, India, in order to improve awareness of NCLs in multiple countries including India.

(2) Development and dissemination of a new diagnostic algorithm for NCLs

As the number of known NCL genes had increased from eight to at least thirteen, a new diagnostic algorithm had to be established for NCLs. A DEM-CHILD diagnostic algorithm for NCL diseases had been developed in collaboration with all NCL clinical experts in the consortium and published as part of a review on clinical presentation of NCL diseases (Schulz A. et al, BBA 2013). Dissemination of this algorithm was performed by different means:

Workshops

Six DEM-CHILD teaching workshops focussing on this diagnostic algorithm have been organised during the entire project period (Table 5). During these workshops, not only the diagnostic algorithm but also performance and evaluation of diagnostic procedures such as electroretinogram (ERG), electroencephalogram (EEG), light microscopy of blood smears (LM), electron microscopy (EM), and cMRI have been taught by the respective specialists. Each workshop has been attended by approximately 80-100 participants such as paediatricians, child neurologists, adult neurologists, care givers, lab technicians etc.

Handouts to medical professionals

A flyer illustrating both, this diagnostic algorithm for NCL diseases as well as providing information where to send diagnostic samples, has been designed and distributed not only during the respective DEM-CHILD teaching seminaries but also at various international congresses (see list of dissemination activities). The flyer can also be downloaded for free from the DEM-CHILD website.

Project website

A project website has been created and can be accessed under http://www.dem-child.eu. The website provides information regarding the new diagnostic algorithm (handout can be downloaded for free), as well as information regarding addresses, contact persons, institutions to where and under which conditions diagnostic samples should be sent in the specific country.

Teaching video

A teaching video on NCL has been created with the support of the German NCL Family Association. For this purpose, German NCL patients gave permission to be recorded during neurologic exams, EEG, MRI etc. when they visited the NCL clinic at the University Medical Center Hamburg-Eppendorf, Germany (partner 01). The video has already been given to NCL families at the Annual Meeting of the German NCL Parents Association in September 2012. Once translated, it will be distributed during the project's next diagnostic workshops (see above) and can also be ordered for free via the project's website.

(2) Development of new testing and screening methods applicable to the wider community

Two WPs (WP1 and WP2) of DEM-CHILD have successfully developed new testing and screening methods applicable to the wider community:

(a) **Development of a diagnostic gene chip**

The novel Next-Generation-Sequencing (NGS) -based diagnostic tool designed and developed by partners 11 and 12 in WP01 has the potential to become a significant extension of the existing gene-based NCL diagnostics. Within the DEM-CHILD funding period we could establish a state-of-the-art technology which will allow, within a few weeks, to scan the currently known NCL related gene loci plus a number of NCL candidate genes base by base. This is a clear improvement of the current situation, where no NCL diagnostic tool like this is available to our knowledge. In contrast to yet existing methods like PCR and/or classical Sanger sequencing, all relevant genomic regions are covered by the new diagnostic tool in one step and with the highest possible resolution. As of today, the NGS-tool is comparable in terns of costs to microarray-based methods, but superior in terms of resolution and precision. Since the NGS-technology is still advancing very fast, it can be expected that the costs will go down further and a price level of less than 500 Euros per sample will be possible very soon. Partners 11 and 12 are able and willing to offer the tool as a full service to all researchers and physicians in the NCL field and the wider community. The service can be delivered from today on, without further technology developments. All necessary equipment, know-how and software tools are available in the partners companies.

In terms of novel gene mutation detection the studies carried out within DEM-CHILD resulted in a significant number of mutations, which were not known before and which are subject to further screenings and studies with the project partners (1,2,3,4,5).

All data generated by the diagnostic tool are and will be available to the project partners via the ftp-servers of partners 1, SME partners 11 and 12 have successfully developed a new genetic testing method for NCLs, which significantly reduces time and costs for diagnosis and which is readily available to the wider community.

(b) Development of automated enzyme testing for CLN1, CLN2, and CLN10

Three forms of NCL are caused by a deficiency in soluble lysosomal enzymes (CLN1, CLN2, CLN10). These three NCL forms can be easily diagnosed using fluorometric enzyme activity measurements in dry blood spots. These assays had already been established before the beginning of the DEM-CHILD project. However, they are quite time consuming, because measurements cannot be automated and single assays have to be run for each of the three enzymes.

Early detection of paediatric neurodegenerative diseases in Europe and India

Even though all NCL diseases are still beyond remedy, for three forms of NCL caused by a deficiency in soluble lysosomal enzymes (CLN1, CLN2, CLN10), significant progress has been made recently in preclinical studies in mouse models of these diseases using a variety of approaches to deliver the missing gene product into the brain. As such there have been relatively successful attempts at enzyme replacement therapy, viral-mediated gene transfer, and more recently neural stem cell transplantation, but these effects are greatest if the therapy is delivered early in disease progression. Based on first promising effects upon behavioural and neuropathological phenotypes, two phase I / II clinical trials for CLN2 disease for the first two approaches are open for enrollment. This demonstrates that therapy options for NCL forms caused by the deficiency of single soluble lysosomal enzymes (CLN1, CLN2, and CLN10) are within reach.

These three NCL forms can be easily diagnosed using enzyme activity measurements in dry blood spots. Dry blood spots are also used for newborn screening for a variety of metabolic diseases. But newborn screening in European countries is only legal, if a treatment option exists for such diseases. Therefore, to date, the NCL diseases are excluded from such newborn screening programs. Once a therapeutic option becomes feasible for the CLN1, CLN2, and CLN10 forms of NCL, the opportunity of newborn screening for these diseases will have to be discussed again, as early detection of the disease will be crucial for the therapeutic success. Taking into consideration that phase I / II clinical trial for one of these diseases, CLN2, have started already, it is crucial to prepare testing methods applicable for future newborn screening efforts once treatment options are available.

The successful development of automated multiplex enzyme activity testing for CLN1, CLN2, and CLN10 by SME partners 7 and 13 (WP02) will ensure, that a method for newborn screening of these diseases is readily available

once therapeutic options do exist. In addition, even before being used for newborn screening, this technique will significantly reduce time and costs for diagnosis, and support early detection of NCL diseases in Europe and India.

In addition, extensive collection and analysis of natural history data of these diseases in WP03 has led to the identification of early risk factors for certain forms of NCL such as CLN2. These will be implemented in targeted population screening programs in order to improve early diagnosis of NCLs This will also have a scientific impact as it will enable the study of prevalence and incidence for these diseases in large patient populations as well detect high risk populations.

In terms of novel gene mutation detection the studies carried out within DEM-CHILD resulted in a significant number of mutations, which were not known before and which are subject to further screenings and studies with the project partners (1,2,3,4,5). All data generated by the diagnostic tool of WP01 are and will be available to the project partners via the ftp-servers of partners 1, 11, 12. This will improve the interpretation of diagnosed sequence variations in NCL genes and reduce the time towards a clear genetic diagnosis for patients and families.

WP7 has contributed the identification of novel mutations, and added to the data on patients with known mutations. This will enable faster diagnosis in families, lowering the risk of multiple children being affected as genetic counselling can be provided and the opportunity to test prenatally to ensure delivery of an unaffected child. Relatives can be tested for carrier status. It has also contributes to knowledge on the genotype-phenotype variability of NCL, as the number of affected individuals has been substantially increased.

Development and validation of new therapies

As called for in the work program of HEALTH.2011.2.2.1-4, the DEM-CHILD project is expected to have a major impact on the creation of tools for experimental therapy in NCL diseases which are currently incurable. Four out of nine work packages of the DEM-CHILD project aim at the development and validation of new tools for experimental therapies for these diseases.

Three out of the nine work packages of the DEM-CHILD project have a significant impact for the development of new rationale therapies for NCLs. One work package has focused on validating therapies in patients

WP4 and **WP5** have studied modifier and biomarker genes of CLN3 and CLN1 disease, respectively, which represent two of the three most common NCL forms in Europe. **WP6** has focused on the development of therapies for NCL forms caused be defects in transmembrane proteins, CLN3 and CLN6. The extensive collection of longitudinal natural history data in **WP3** represents an important tool for the evaluation of experimental therapies in patients.

<u>WP4: Biomarkers and modifiers of CLN3</u>

WP4 has contributed to future development of therapies for CLN3 disease by

(1) extending the broad spectrum of model organisms available for studying CLN3, which will enable better understanding of gene function and disease mechanism;

(2) identifying a novel signalling pathway and potential therapeutic targets, together with three small molecules in the yeast model

(3) identifying regulatory components and mechanisms capable to alter the gene expression profile in CLN3-defective mouse brain cells;

(4) evaluating the dual specificity phosphatase 2 (DUSP2) as a highly potential biomarker of CLN3 disease

(5) performing the first comprehensive analysis of the lysosomal CLN3 proteome which identifies subsets of lysosomal enzymes impairing lysosomal homeostasis and lysosomal targeting routes that might represent novel targets for therapeutic approaches.

These results will provide important targets for future alternative therapeutic strategies that lead to the development of new treatments.

<u>WP5: Biomarkers and modifiers in CLN1</u>

WP5 characterized molecular basis and pathophysiology in CLN1 cell and animal models with the goal to identify modifiers and biomarkers of CLN1. WP5 has utilized induced pluripotent stem (iPS) cells from CLN1 patients to show that CLN1 has a role in neuronal extension and migration. WP5 could also show that enhanced apoptotic cell death

and defects in mitochondrial function are part of the CLN1 cell pathology. Furthermore, results derived from proteomic profiling using MALDI-MS in CLN1 knockout mouse brain tissue showed that - depending on the disease stage - different metabolic processes in specific brain structures are affected. The findings of WP5 indicate that CLN1 disease has a major neurodevelopmental component and therapeutic trials will have to be performed very early in life.

The results of DEM-CHILD WP5 regarding CLN1 disease modifiers and biomarkers as targets for future therapies will have a clear impact on future therapeutic strategies of CLN1 disease since in this programme we have confirmed two defective pathways - originally detected in the *Cln1/Ppt1-/-* mouse model – now also in human iPSderived neurons as well as human CLN1 fibroblasts. Proteomic profiling further confirmed the earlier results obtained in the mouse model by other methods. Altogether, the future research programmes have now the possibility to follow-up certain metabolic pathways defective in the CLN1 disease. Currently, the major treatment studies are performed in the mouse model and this study provides clear advances to follow up the treatment of mouse models. However, the fact we could here show for the first time that some of the CLN1 defects observed in the mouse model replicated also in the human CLN1 cells, will open up a new avenue to develop imaging and spectroscopic technologies to follow up the outcome of treatment in the human patients.

WP 6: Innovative therapies for NCLs caused by mutations in transmembrane proteins

The central aim of this WP6 was to develop novel therapies for the treatment of NCL caused by deficiency in transmembrane proteins. In contrast to NCL forms caused by a deficiency in a soluble lysosomal protein (CLN1, CLN2, CLN10), where significant progress has been made recently in preclinical and clinical studies using a variety of approaches to deliver the missing gene product into the brain, to date the therapeutic outlook for those individuals with mutations in transmembrane proteins still remains very bleak. In addition, the number of patients being diagnosed with NCL forms caused by defective NCL-related transmembrane proteins (CLN3, CLN6, CLN7, and CLN8) is increasing. Therefore, there is a significant and unmet need to embark on the challenging task of developing therapies for these NCL forms.

In order to address this need, WP6 has investigated the feasibility of two therapeutic protocols in mouse models of disease:

1. AAV-mediated delivery of the missing CLN6 or CLN3 genes to the retina and brain, respectively, and
2. neural stem cell-mediated delivery of neuroprotective factors to the retina

The **retinal *CLN6* gene therapy study** has shown that gene supplementation of the *CLN6* gene to the mouse photoreceptor cells is insufficient to attenuate photoreceptor cell degeneration. Future experiments will aim to target the bipolar cells specifically.

The ***CLN3* gene therapy study** analysed the delivery of CLN3 to the CNS showing that AAV2/9 vectors are capable of widespread and long lasting transduction of the CNS.

The **study using stem cell grafts to deliver neurotrophic factors to the retina** has shown that expression of CNTF can attenuate the loss of the photoreceptor cells in the nclf (CLN6) mice. These results encourage further research into a factor that rescues the photoreceptor cells without negatively affecting their function.

Taken together, WP6 has a major impact on the challenging subject of the development of therapies for NCL diseases caused by mutations in intracellular transmembrane proteins, in particular the juvenile CLN3 and the variant late infantile CLN6 disease.

WP3: Epidemiology and natural history of NCLs

The NCL Registry (WP3) will have a significant impact on the validation of current of future experimental therapy studies in NCLs. To date the DEM-CHILD NCL patient database contains the largest set of natural history data for all NCL forms worldwide which will continue to grow with the participation of the new database consortium members from a total of 12 countries. Results from the analysis longitudinal natural history data describe well that data derived from the DEM-CHILD NCL patient database represent a valid tool for the evaluation of current and future experimental therapy studies. In fact, CLN2 natural history data collected in WP3 are already used as control data in a phase I/II clinical trial on intraventricular enzyme replacement therapy in CLN2 disease.

As to date there is still no cure for any form of NCL, palliative treatment and supportive care are an important part of current treatment in all NCL forms. Therefore information on current medication to treat the most severe symptoms in all NCL forms such as epilepsy, myoclonus and spasticity has been collected in the DEM-CHILD database including

the respective positive and negative effects, analysed and published. These data will help to improve palliative medical care for NCL patients and thus help to reduce the burden of disease and improve quality of life in these patients. In addition, this approach will also enhance the delivery of cost-effective care, helping to reduce the costs of health care provision.

International cooperation
European cooperation of DEM-CHILD

The DEM-CHILD project has proven that working at a European level in the field of the NCLs is indispensable:

Since the NCLs are complex disorders, caused by defective soluble lysosomal enzymes or defects in transmembrane proteins, affecting both the retina and brain, there is an increasing need to concentrate research efforts by reaching a critical mass of complementary competencies (genetics, neurology, child neurology, paediatrics, ophthalmology, molecular biology, biochemistry etc) that are spread out around Europe, creating a real synergistic effect from a technical and financial point of view. Without this concentrated effort, there would not be such synergy, which would subsequently slow down research and considerably delay the discovery of new treatments and their delivery to patients. Therefore, in all basic research work packages (WP4, WP5, and WP6), several DEM-CHILD partners from different European countries have collaborated within the same work package. Exchange of data, mouse models, research samples has been routinely implemented without any problem. Moreover, PhD students were given the opportunity to spend a certain time in another laboratory to learn new research techniques. Moreover, using the "virtual biobank" implemented in the DEM-CHILD NCL patient database, exchange of patient material for different research projects has become a routine within the consortium and beyond.

Since the NCLs are rare disorders, studying a significant number of patients is a crucial point to gain statistically significant data in order to accurately understand each NCL form and its natural course. Because the prevalence of the different NCL forms varies in different European countries, only a collaborative effort of several European countries can guarantee that enough NCL patients of each NCL form are studied. Therefore, all partners pf the DEM-CHILD project (Germany, UK, Italy, Finland) have successfully collaborated in WP3 in extensively collecting natural history data on all NCLs and in establishing prevalence figures.

Close cooperation with India and Europe

The cooperation between the European partners and the Indian partner has been very close throughout the entire DEM-CHILD project despite the fact that the project had to overcome some challenging regulatory obstacles:

Due to the late joining of our Indian partner in month 13 of the project and due to a restriction that only 10% of patient material was allowed to leave India and to be entered into the project, the number of samples (n=63) the project has received so far for diagnosis is too small to be used for any prevalence studies for India. The 10% restriction rule had been enforced by the Indian Council of Medical Research (ICMR) after the project started and was a pre-condition that partner 05 got permission from the ICMR to sign the DEM-CHILD grant agreement and be part of the consortium. The new restriction rule for India had been included in amendment no. 1 and accepted by the European Commission.

In order to face this challenge, we have tried to overcome these difficulties in obtaining reliable prevalence figures for India by increasing diagnostic capabilities for India: All partners have developed independent contact to Indian clinicians not connected with this project, and to clinicians of other countries in which Indian families were living. Moreover, we have organised one of our DEM-CHILD teaching workshops during the Annual Meeting of the Association of Child Neurology in Hyderabad, India, where also flyers with the newly developed diagnostic algorithm were distributed. Since then the number of samples transferred for diagnosis has increased, especially during the 4 weeks after project end (n=45). For those samples, diagnosis is still pending.

To conclude, the cooperation with India has achieved to improve awareness and diagnosis of NCLs in India despite national regulatory challenges.

Cooperation with other international research activities

In order to extensively study the natural course and clinical spectrum of all NCL forms, the DEM-CHILD database consortium has expanded to a total of twelve participating countries by now (Germany, UK, Finland, Italy, France, Norway, Denmark, Turkey, India, US, Brazil, Argentina) with the aim to collect the world's largest, clinically and genetically best characterized, set of NCL patients. Funding for the new partners Norway, Denmark, France, Turkey,

Brazil, and Argentina was obtained through two ways: i) a successful research grant application to the American NCL Family Association BDSRA (153 000 USD) coordinated by partner 1; ii) financial compensation for transfer of anonymous natural history data of CLN2 patients collected by DEM-CHILD partners to the company BioMarin to serve as historical control data for a phase 1 enzyme replacement study for CLN2 disease (100 000 €). The DEM-CHILD consortium had voted unanimously to use the entire newly obtained funding to finance database managers for each new partner country of the DEM-CHILD Database Consortium.

Patient numbers provided from all current DEM-CHILD database partners from the twelve participating countries add up to a total of 558 NCL patients

Participant 3 who coordinates the International Rare NCL Gene Identification Consortium (RNGC), offered the research opportunity offered by DEM-CHILD to identify new genes to clinicians in the RNGC as an encouragement to submit new families. In addition, participant 3 became involved in a novel Consortium focusing on adult onset NCL, although in this case little of this work was directly relevant to DEM-CHILD.

Boosting the innovative capacity of European health-related industries (SME participation)

Strong SME participation

At the beginning of the project, the DEM-CHILD consortium involved three SMEs. During the project, additional three SMEs have been added in order to boost the innovative capacity of several work packages.

Two WPs highly depended on the innovative work of SMEs, WP1 and WP2 which should develop new diagnostic tools for the consortium.

In **WP1** there was and is a close cooperation between SME partners 6,11,12 and the academic partners 1,2,3,4 and 5. It turned out that the flexibility and expertise of the SMEs was ideal for the challenges and needs of the project, e.g. the adoption of essential methods and even changes of technologies within project run time was completely managed by the SMEs.

In **WP2**, SMEs partners 7 and 13 successfully developed the multiplex automated enzyme assay for CLN1, CLN2, and CLN10.

SMEs (partners 6,11,12,) were also very involved in the basic research projects of **WP4,** and **WP5** (detection of biomarkers) where they provided technique and expertise for large-scale egene expression analyses.

Also **WP7** has benefited a lot from the capabilities, which SMEs (partners 11,12) can offer by the diagnostic tool developed in WP1, since the classification of NCLs needs a strong and differential diagnostics.

The SME GABO:mi has led **WP9** and taken full responsibility for contractual and communication management, project controlling and reporting, finance controlling and reporting, meeting organisation, and monitoring of IPR regulations. This expertise in project management has ensured that the DEM-CHILD project has not encountered problems due to mismanagement in any of these areas and that researchers had sufficient support in these areas so that they could focus on their research activities.

Innovation through SME participation

The SMEs partners 6,11,12) in WP01 developed a new sequence-based diagnostic tool, which offers for the first time a routine application, i.e. detection of yet classified NCL-relevant mutations, plus screening of mutations in a number of potentially relevant genes (candidate genes). The diagnostic tool in its present form did not exist before and will allow a much better genetic diagnostics of NCLs and also help a lot to find new markers (WP04, WP05) and even mutations (WP07) not yet known before. The SMEs partners 11,12) have the infrastructure and technologies in place to offer the NCL gene-based diagnostics to project partners and all interested researchers and physicians,

The SMEs partners 7 and 13 have successfully developed a MS-based automated multiplex enzyme for CLN1, CLN2, and CLN10. Such multiplex assay has never existed before and will significantly reduce time and costs for diagnosis of these NCLs.

These tools will not only be used by the DEM-CHILD project, but are now available to clinicians in general. These diagnostic tools significantly reduce time and costs for the diagnosis of all NCL forms and therefore have a major impact on the prevention and early detection of these diseases worldwide.

Main dissemination activities and exploitation of results

WP01: Development of a diagnostic gene chip

The diagnostic tool can be used as is by NCL researchers and physicians after the funding period, a first concepts of commercialization is under discussion. It was agreed between the partners that the companies of partners 11 and 12 are well prepared and are willing to offer the tool as a full service to the NCL community. A respective price model will be negotiated shortly. For partners 11 and 12 the tool will be a service component, which is well integrated in their yet existing facilities.

There is strong interest by the project partners to continue the use of the NGS-based diagnostic tool after end of DEM-CHILD and the SMEs (11,12) are supporting the initiatives to make the tool available to the world. Especially in terms of finding novel biomarkers and yet unknown NCL-relevant mutations the diagnostic tool is planned to be used by the partners after end of DEM-CHILD.

WP02: Automated enzyme analysis for CLN1, CLN2, and CLN10

Similar to WP01, this new diagnostic tool can be used as is by NCL researchers and physicians after the funding period, a first concepts of commercialization is under discussion.

Outlook and future research

WP01: Development of a diagnostic gene chip

NCL diagnosis will benefit significantly from the possibility to perform deep and comprehensive genetic testing (sequencing) of patients within a very short time period (a few weeks). In addition, the diagnostic tool will allow a quick and substantial scan of yet undetected mutations and their relation to NCL. The more analysis runs are performed the more the findings of identical mutations will increase their score to be real and to be related to NCL. Similar tools based on target sequencing are currently developed for a number of disease loci, so that we believe that our NCL diagnostic tool will be built on state-of-the-art technology for the next years.

WP2 Automated enzyme testing for CLN1, CLN2 and CLN10

The newly developed MS-based multiplex enzyme assay will be used for targeted populations screening in order to pbtain incidence figures for NCLs in various countries.

Moreover, the new diagnostic tool will be made available to the international community for rapid and cost-effective diagnosis of CLN1, CLN2, and CLN10.

WP3 Epidemiology/ Natural history study

Work of the DEM-CHILD NCL database consortium will continue beyond the DEM-CHILD project time. As previously indicated, the DEM-CHILD database consortium has expanded to a total of twelve participating countries by now (Germany, UK, Finland, Italy, France, Norway, Denmark, Turkey, India, US, Brazil, Argentina) with the aim to collect the world's largest, clinically and genetically best characterized, set of NCL patients. Funding for the new partners Norway, Denmark, France, Turkey, Brazil, and Argentina was obtained through two ways: i) a successful research grant application to the American NCL Family Association BDSRA (153 000 USD) coordinated by partner 1; ii) financial compensation for transfer of anonymous natural history data of CLN2 patients collected by DEM-CHILD partners to the company BioMarin to serve as historical control data for a phase 1 enzyme replacement study for CLN2 disease (100 000 €). The DEM-CHILD consortium had voted unanimously to use the entire newly obtained funding to finance database managers for each new partner country of the DEM-CHILD Database Consortium.

Patient numbers provided from all current DEM-CHILD database partners from the twelve participating countries add up to a total of 558 NCL patients

WP4 Biomarkers and modifiers of CLN3

The work using yeast will provide background for new applications for further work on understanding CLN3 gene function and developing new therapies. The studies on the dysregulated gene network and the lysosomal protein composition in CLN3-defective brain cells will provide new insights into pathogenesis and modulatory processes affecting the course of the disease which might be novel targets for therapeutic approaches.

WP5 Biomarkers and modifiers of CLN1

The results of DEM-CHILD WP5 regarding CLN1 disease modifiers and biomarkers as targets for future therapies will have a clear impact on future therapeutic strategies of CLN1 disease since in this programme we have confirmed two defective pathways - originally detected in the *Cln1/Ppt1-/-* mouse model – now also in human iPSderived neurons as well as human CLN1 fibroblasts. Proteomic profiling further confirmed the earlier results obtained in the mouse model by other methods. Altogether, the future research programmes have now the possibility to follow-up certain metabolic pathways defective in the CLN1 disease. Currently, the major treatment studies are performed in the mouse model and this study provides clear advances to follow up the treatment of mouse models. However, the fact we could here show for the first time that some of the CLN1 defects observed in the mouse model replicated also in the human CLN1 cells, will open up a new avenue to develop imaging and spectroscopic technologies to follow up the outcome of treatment in the human patients.

WP 6 Innovative therapies for NCLs caused by mutations

The analysis of treatment outcome after AAV2/9-mediated gene delivery to the brain of the CLN3-deficient mice is still ongoing, but the level, spread and longevity of CLN3 expression show promise for the future. If a positive effect of treatment on neuronal survival is found, further research will be needed firstly to resolve the variation in transduction efficacy that was detected and secondly to compare the efficacy of treatment in the striatum with that in other CNS structures, as part of the process of translating this work to the clinic. Furthermore, the identification of AAV2/9 as an efficient vector for gene delivery to the CNS also promotes gene therapy as a promising treatment approach for a range of recessive disorders of the brain, including other transmembrane (and soluble) forms of NCL, other lysosomal storage diseases, and certain forms of Parkinson's disease.

Despite considerable effort, the development of retinal gene therapy for CLN6-deficiency has thus far been failed to show a positive treatment effect. However, the data does provide us with pointers for future studies. The fact that a successful supplementation of the CLN6 gene to the photoreceptor cells at an early age fails to provide a benefit suggests that the photoreceptor cells are not the primary cell type affected, or not the only retinal cell type affected by the disease. This conclusion is supported by new data that show high levels of CLN6 protein in the inner nuclear layer, probably the bipolar cells, in the human retina. Future studies aiming to treat this condition should therefore focus on the delivery of CLN6 to the bipolar cells, either in isolation or in combination with transduction of the photoreceptors.

The use of stem cell grafts to deliver neurotrophic factors to the retina has shown that expression of CNTF can attenuate the loss of the photoreceptor cells in the Cln6nclf mice for at least 6 weeks. As CNTF delivery has failed to be efficacious in a clinical trial for the treatment of retinitis pigmentosa, there are some doubts whether the work is directly applicable in the clinic. However this study does show that the photoreceptor cells in the CLN6 retina are responsive to sustained exposure to a neurotrophic factor and encourages further research into a factor or combination of factors that rescues the photoreceptor cells without negatively affecting their function. Moreover, as the principle behind the method is independent of the disease mechanism and target cell, the results can help in the development of similar therapeutic approaches for other retinal dystrophies and potentially other neurodegenerative diseases.

WP7 Identification of new NCL genes

The data on prevalence for each NCL type in Europe will influence the priority of therapeutic development for these individual diseases. For example, enzyme replacement therapy for CLN2 disease is in phase 1 clinical trial, and lays the groundwork for similar therapy for other NCL forms caused by mutations in soluble lysosomal enzymes - CLN1, CLN10, CLN13. However, the most common type of NCL in Europe is CLN3 disease. CLN6 and CLN7 diseases are more common in Mediterranean countries and were a significant portion of Indian families that were genetically identified. These three genetic types are caused by mutations in membrane proteins, which are beyond current therapeutic technologies. Work in DEM-CHILD on CLN3 disease, and the use of the mouse model for CLN6 disease for the development of retinal gene therapy, has inspired the formation of a new consortium, BATCure, in response to a Horizon20:20 call that involves three participants of DEM-CHILD with novel partners, to work towards new therapies for these very challenging NCLs.

WP8 Dissemination

Many children in India in whom NCL was neither suspected nor diagnosed have been examined for the disorder. Several of them have also been diagnosed with NCL through the project partners. This has clarified that all types of NCL are prevalent in India but are not really being diagnosed. The creation of the genetic analysis and hopefully its

availability at a reasonable price in the near future is expected to help in the genetic diagnosis of NCL in India in a big way.

The project has also opened gateways for further international research. Younger scientists from India would be able to find research positions with various EU partners in future. Also international researchers interested in NCL would be assured that there is a lot of potential in India to conduct research on these disorders.

The DEM-CHILD database consortium will continue its work and also continue to update the DEM-CHILD webiste and diagnostic algorithm in order to ensure and improve awareness of NCL disease internationally.

Section 2 – Use and dissemination of foreground

2.1 Plan for use and dissemination of foreground (including socio-economic impact and target groups for the results of the research)

Section A

List of scientific publications

The following scientific publications are in preparation but not published yet:

WP02: Automated enzyme analysis for CLN1, CLN2, and CLN10

The following manuscript is in preparation

- Przybylski M et al. Development of a triplex assay for neuronal ceroid lipofuscinoses CLN1, CLN2, and CLN10 by tandem mass spectrometry (MS-MRM)

WP03: Epidemiology and natural history

Publications related to this work package:

- Schulz A et al. NCL diseases – Clinical Perspectives. Biochem Biophys Acta 2013; 1832(11): 1801-1806.
- Santorelli FM et al. Molecular epidemiology of childhood neuronal ceroid lipofuscinosis in Italy. Orphanet J Rare Dis 2013: 8:19.
- Schulz A et al. Neuronale Zeroidlipofuszinsen: Metabolische Demenz-Krankheiten im Kindesalter. Monatsschr Kinderhlkd 2012: 734-741.
- Bergholz R et al. Phenotyping heterozygous carriers of juvenile neuronal ceroid lipofuscinosis with *CLN3* mutations Graefes Arch Clin Exp Ophthalmol 2014: in press.
- Kohlschütter A et al. Epielepsy in Neuronal Ceroid Lipofuscinoses. J Pediatr Epilepsy 2014, in press
- Kohlschütter A et al. Demenzerkrankungen bei Kindern und Jugendliche, Pädiatrische Praxis, 2014, in press.

The following manuscripts are in preparation:

- The DEM-CHILD database consortium: The DEM-CHILD NLC patient database – a tool for the evaluation of experimental therapy studies.
- Schulz A et al. The Natural History of Late Infantile CLN2 Disease: Striking Homogeneity of Clinical Progression in Two Independently Obtained Large Clinical Cohorts.
- Loebel U. et al. Longitudinal MRI Volumetry of Gray Matter in Neuronal Ceroid Lipofuscinosis Subtype CLN2 Reveals a Unique Uniformity of Disease Progression.
- Nickel M et al. Late Talkers in late infantile CLN2 disease: Red flags for an early diagnosis.
- Simonati A. et al. Analysis of genotype-phenotype variability in Italian CLN5 patients.
- Moll-Khosrawi P. et al. Cardiac involvement in NCL diseases.

WP04: Modifiers and biomarkers of CLN3

Publications related to this work package:

- Kollmann K et al. Cell biology and functions of neuronal ceroid lipofuscinosis related proteins. Biochem Biophys Acta 2013; 1832(11): 1866-1881.

The following manuscripts are in preparation:

- Bond M et al. Genome-wide analysis reveals a central role for Tor signalling in a yeast model for neurodegeneration.

- Schmidtke C et al. *Cln3*$^{Dex7/8}$ impairs endocytic receptor trafficking and efflux of lysosomal amino acids.
- Makrypidi G et al. TFEB control through ERK signaling by Dual Specificity Phosphatase 2.

WP5 Biomarkers and modifiers of CLN1

Publications relating to this work package

- Tikka S et al. Proteomic profiling in CLN1 disease brain: an imaging and label-free proteomics approach. PloSBiology 2014, in press
- Skifo E et al. Proteomic Analysis of the Palmitoyl Protein Thioesterase 1 Interactome in SH-SY5Y Human Neuroblastoma Cells. J Proteome Res 2014, in press.

Additional results of WP5 will be published in international journals and the data has been presented in several international congresses (see dissemination activities in ECAS.)

WP 6 Innovative therapies for NCLs caused by mutations

Publications relating to this work package:

- Bartsch U et al. (2013) Apoptotic photoreceptor loss and altered expression of lysosomal proteins in the nclf mouse model of neuronal ceroid lipofuscinosis. Invest. Ophthalmol. Vis. Sci., 54:6952-6959.
- Sondhi D et al. (2014) Partial correction of the CNS lysosomal storage defect in a mouse model of juvenile neuronal ceroid lipofuscinosis by neonatal CNS administration of an adeno-associated virus serotype rh.10 vector expressing the human CLN3 gene. Hum Gene Ther., 25:223-239

In the latter study, immunohistochemistry analysis of brain sections was performed by DEM-CHILD partner 08.

A manuscript on the outcome of the stem cell mediated delivery of CNTF is under revision:

- Jankowiak W et al. Sustained neural stem cell-based intraocular delivery of CNTF attenuates photoreceptor loss in the nclf mouse model of neuronal ceroid lipofuscinosis. In revision.

Two manuscripts on the AAV-mediated gene therapy for the CNS in the Cln3 mouse and for the retina in the Cln6 mouse are in preparation. Additionally, study results have been disseminated to a scientific audience through presentations and posters at scientific meetings, and to a lay audience through presentations to patient/family organisations and seminars at joint academic – patient meetings.

WP7 Identification of new NCL genes

Publications relating to this work package:

- Bras J et al. Mutation of the parkinsonism gene ATP13A2 causes neuronal ceroid-lipofuscinosis. Hum Mol Genet 2012: 21:2646-2650.

Many of the results of WP7 have been incorporated into the NCL mutation database, and any received beyond the end of the project will be added immediately. They will also be included in a publication summarising the overall results on all families submitted to this project, in an effort that completes the spectrum of NCL disease. This manuscript is in draft form, and awaiting completion of the final analysis before submission for publication.

For more information on already published publications please see the ECAS system.

Section B

No patents, trademarks, registered designs, etc. were applied.

Section 3 – Report on societal implications

Please see ECAS.