

Use of an MLPA-based approach for an initial and simultaneous detection of GBA deletions and recombinant alleles in patients affected by Gaucher Disease

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Gaucher disease (GD), the most common lysosomal storage disorder, results from mutations in GBA gene. To date, more than 430 different mutations have been described [<http://www.hgmd.org>] including single base-pair substitutions, deletions, insertions in GBA gene and complex rearrangements between GBA and its highly homologous pseudogene (pGBA).

In this study, we have set up a Multiplex Ligation-dependent Probe Amplification (MLPA)-based approach (MRC-Holland, P338-X1) to investigate the potential occurrence of large GBA deletions.

Overall, we have analysed 88 alleles belonging to 44 individuals of whom 26 were patients affected by GD and the remaining 18 were unrelated healthy controls. Among GD patients, 19 had a still-uncharacterised allele whereas 7 patients were already known to carry complex recombinant alleles (RecNcil, delta 55 and Complex I-rec7) or common point mutations such as L444P and F213I.

The samples with known mutations were primarily included in the analysis to evaluate the reliability of MLPA-based approach in discriminating GBA from pGBA sequences.

Overall, we have identified 3 novel alleles belonging to 5 patients: 2 partial gene deletions (3-9 exon del and 7-10 exon del) and a recombinant allele involving GBA-GBAp genomic sequences between exons 6 and 9. In addition, we have also identified a large deletion already described by Cozar et al. (2011)1.

In conclusion, based on these preliminary results we consider MLPA approach as an effective tool for an initial detection of the genomic sites where deletions and recombinant alleles approximately occur, but we strongly recommend confirmation of any finding and the precise characterization of the mutant alleles by conventional methods.

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Preferred presentation
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Identification of molecular targets for the treatment of the skeletal phenotype in Lysosomal Storage Disorders

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Lysosomes are the major catabolic organelles within the cell, being central for degradation and recycling of macromolecules delivered by endocytosis, phagocytosis, and autophagy. Mutations in genes encoding for lysosomal proteins cause Lysosomal Storage Disorders (LSDs), a class of inborn pathologies characterized by the accumulation of material in lysosomes (1). The massive accumulation of substances affects the function of lysosomes, resulting in secondary inhibition of lysosome-related processes, such as impairment of autophagy and endocytosis (2). More recently, the lysosomes emerged as crucial regulators of cell homeostasis being platforms for the regulation of mechanistic target of Rapamycin kinase complex 1 (mTORC1) signaling in response to nutrients (3). However, an involvement of mTORC1 in the pathogenesis of LSDs is still unknown. Here we show that alteration of mTORC1 signaling contributes to bone growth retardation in Mucopolysaccharidosis type VII (MPS VII), a severe model of LSD (4). Primary chondrocytes isolated from MPS VII mouse model showed enhanced mTORC1 activity in response to nutrient due to persistent association of mTORC1 to lysosomes. Both genetic and pharmacological limitation of mTORC1 signaling rescues autophagy dysfunction in MPS VII chondrocytes. Furthermore, genetic limitation of mTORC1 signaling rescued chondrocyte activities and long bone growth retardation in MPS VII mouse model. These data highlight the physiological role of lysosome as regulator of mTORC1 signaling during bone development and suggest the pharmacological modulation of mTORC1 as therapeutic approach for the treatment of skeletal phenotype in LSDs.

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Preferred presentation

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Is peripheral neuropathy a constitutive element of the non-neurological phenotype of Gaucher disease?

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Introduction. Type I Gaucher (GD1) disease is a lysosomal storage disorder usually differentiated from types II and III Gaucher disease by the absence of nervous system involvement. However, an increasing number of reports has recently described neurological manifestations including subclinical large fibre neuropathy. Pain is one of the more disabling symptoms in GD1, often persisting despite enzyme replacement therapy (ERT). It is currently considered as nociceptive pain secondary to bone involvement, but it is described even in absence of the latter without explanation. **Aim of the study.** To investigate the presence and characteristics of pain in a cohort of GD1 patients and to assess the peripheral nerves involvement. **Material and methods:** 25 GD1 patients (13 female, 12 male; 23 on ERT \geq 5 years, 2 naive), followed at the RCCRD were enrolled in the study in the period 2012-2014. **Inclusion criteria:** a) enzyme and molecular analysis; b) age 16-60 years; c) ERT \geq 5 years or naive. **Exclusion criteria:** presence of co-morbidities or biochemical abnormalities related to peripheral neuropathy. Patients' examination included pain questionnaires (Douleur Neuropathique en 4 questionnaire (DN4) and Neuropathic Pain Symptom Inventory (NPSI), clinical and neuroalgological evaluation, sensory profile assessment by quantitative sensory testing (QST) battery, nerve conduction studies (NCS) and skin biopsy at distal leg (DL) and proximal thigh (Pth). Pain intensity and qualities were recorded. **Results:** 12/25 patients reported chronic pain. No one had had bone crisis in the last year. All of them reported painful sensations suggestive of neuropathic pain with proximal patchy distribution, 6 with severe pain paroxysmal. Pinprick hypoesthesia was found in 9 and thermal hypoesthesia in 17. The sensory profile at QST battery showed a sort of stereotypical pattern with high cold thresholds with errata sensation (burning instead of cold), presence of paradoxical heat sensation (PHS) and mechanic hypoesthesia MDT, while only in 3 pressure pain hyperalgesia (PPT) was present. Skin biopsy showed epidermal denervation in 19 patients, in 12 with a non length-dependent pattern. In 10 of them we found only skin denervation at proximal site compared with 96 age and sex match controls. The conventional nerve conduction studies showed only mild L5 radiculopathy in 2 and Carpal Tunnel Syndrome in 1. **Conclusion:** the study assessed for the first time the presence of neuropathic pain in Gaucher disease type 1 due by small nerve fibres impairment. The presence and distribution of the sensory symptoms and signs and skin denervation in most of GD1 patients is consistent with small fibre neuronopathy, in some of them subclinical and probably not responsive to ERT.

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Validation of an NGS-panel for routine diagnosis of lysosomal and peroxisomal disorders

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Background: Today's routine diagnosis of lysosomal and peroxisomal disorders is highly demanding regarding resources, leading to long response times. Using a Next Generation Sequencing (NGS) panel covering all the known genes involved in lysosomal and peroxisomal disorders as a screening tool would improve time- and cost efficiency of the diagnostic process.

Methods: 20 patients with lysosomal or peroxisomal disorders and one commercial reference control were included in the validation. Pathogenic mutations or deletions were previously detected with Sanger sequencing in all patient samples at diagnosis. The custom made panel targets exons and flanking regions of 90 genes. Library construction was done using the SureSelectQXT from Agilent. Prepared libraries were sequenced on the MiSeq instrument (Illumina®). Secondary analysis was performed on-instrument with MiSeq Reporter and variants were filtered with Illumina Variant Studio. Variants of interest were filtered according to allele frequency, exonic/splice site location, phred quality score of ≥ 30 , exclusion of synonymous variants and autosomal recessive or X-linked pattern of inheritance.

Results: High quality data was obtained with excellent coverage for all targeted regions (20x target coverage of 100% and an average coverage of ca 500x). Expected pathogenic mutations (SNVs and small insertions/deletions) were found in 20 of 20 patients. In the commercial reference containing 281 variants, the panel showed a sensitivity of 100% and a specificity of $> 97.5\%$. The high coverage obtained in the sequencing step allowed us to detect the 30kb deletion in the GALC gene in 3 patients with Krabbe disease.

Conclusion: Our NGS-panel will be a useful tool for the detection of pathogenic mutations in lysosomal and peroxisomal disorders, thereby by-passing the diagnostic odysseys that these patients often go through before they get their diagnosis. All positive findings will be confirmed with Sanger sequencing and available biochemical analysis.

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Cerebral lysosomal dysfunction and impaired autophagy in a novel mouse model deficient for the lysosomal membrane protein Cln7

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CLN7 disease is an autosomal recessive, childhood-onset neurodegenerative lysosomal storage disorder caused by the deficiency of the lysosomal membrane protein CLN7. We have disrupted the Cln7/Mfsd8 gene in mice by targeted deletion of exon 2 generating a novel knockout (KO) mouse model for CLN7 disease which genetically resembles CLN7 patients with variant late-infantile phenotype and recapitulates key features of human CLN7 disease. Cln7 KO mice were viable and fertile, but they had increased mortality and a neurological phenotype shown by hindlimb claspings, myoclonus, ataxia and tremor. Lysosomal dysfunction in Cln7 KO mice was detected by the presence of autofluorescent lipopigments mainly in neuronal cells and the accumulation of subunit c of mitochondrial ATP synthase (Scmas) and saposin D in brain tissue. Storage of Scmas in peripheral organs was most pronounced in heart cardiomyocytes and renal tubuli. Ultrastructural analysis of the storage material revealed curvilinear and fingerprint profiles in cerebellar Purkinje cells. Quantitative real-time PCR, immunoblot and enzyme activity analyses revealed increased expression of several soluble lysosomal enzymes in the brain of Cln7 KO mice on the transcriptional and protein levels starting by 5 months of age. Immunohistochemical costainings showed increased levels of CtsZ both in Cln7-deficient microglial cells and neurons. Neuroinflammatory changes with strong astrocytosis and microglial activation starting by 5 and 7 months of age, respectively, were observed in Cln7 KO brains. Magnetic resonance imaging of Cln7 KO brains revealed neurodegeneration in the cerebral, cerebellar cortex and olfactory bulb. Increased levels of LC3-II, the autophagy adaptor p62 and polyubiquitinated proteins were observed in different brain regions of Cln7 KO mice suggesting that impaired macroautophagy represents a major pathomechanism in CLN7 disease. We propose that loss of the putative lysosomal CLN7 transporter in the brain leads to lysosomal dysfunction which impairs constitutive autophagy promoting neurodegeneration.

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Analysis of the accelerating mutation S228F in H⁺/Cl⁻ exchanger CIC-7

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The lysosome is part of the endosomal network and a highly acidic (pH 4-5) cell organelle which contains about 60 different types of acid hydrolases. These are digestive enzymes and have the main function of degrading extra- and intracellular material [1]. The acidification of the lysosome is generated by the pumping of protons into the lumen by V-type ATPases [2]. To reach a low pH, counter-fluxes where both anions like chloride and cations could play a major role, have to be present.

The CLC family of anion transporters contains nine members that function as voltage-gated chloride channels or H⁺/Cl⁻ exchangers [3]. One member of this family, CIC-7, is a slowly voltage-gated H⁺/Cl⁻ exchanger ubiquitously found in lysosomes, where it is the primary chloride pathway, and at the ruffled border of osteoclasts. Knockout mice of CIC-7 or its beta-subunit Osteopetrosis-associated transmembrane protein 1 (Ostm1) display a severe osteopetrosis, a NCL-like lysosomal storage diseases and die within the first six weeks of life [4-6]. Mutations in the encoding gene CLCN7 also underlie osteopetrosis in human patients. Surprisingly, not all of these mutations lead to a loss of function of CIC-7, but some even increase the gating kinetics in a heterologous expression system [7]. We have recently found an accelerated mutant in osteopetrotic cattle that is expressed at normal levels and correctly localized to lysosomes in vivo [8], pointing towards a physiological importance of the slow gating.

We have now generated a knock-in mouse model with the accelerating CIC-7 mutation S288F that is found in human osteopetrosis patients. The investigation of this mouse model, which shows osteopetrotic phenotype will help to elucidate the role of the gating kinetics on the physiology of lysosomes and bone resorption, and to better understand the pathomechanism upon ion transport dysfunction.

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Preferred presentation

I only want to present a POSTER

Ripk3 deficiency does not modify the natural course of murine Globoid cell leukodystrophy or Sandhoff disease

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Globoid cell leukodystrophy (GCL), also known as Krabbe disease, and Sandhoff disease are inborn errors of metabolism caused by dysfunction of the lysosomal enzymes β -galactocerebrosidase (GALC) and β -hexosaminidase (HEXA and HEXB), respectively. Whereas GALC hydrolyses the myelin-rich lipid galactocerebroside, HEXA is essential for GM2 degradation, a neuronal ganglioside. Disease-causing mutations in genes GALC and HEXB disrupt the normal workings of the nervous system with catastrophic consequences.

Loss of myelin-producing cells in GCL and neurones in Sandhoff disease has been reported, but the underlying molecular mechanism of their demise has not been dissected out. Recent findings by Vitner and colleagues implicated key components of necroptosis, receptor-interacting protein kinases 1 (Ripk1) and 3 (Ripk3), as active contributors to neuropathic Gaucher's disease, also a lysosomal disease. Significantly, when Ripk3 knock-out mice were induced to develop Gaucher's disease, Ripk3 deficiency protected the animals - increased survival concomitant with delayed disease signs was notable.

Patients and animals suffering from neuropathic Gaucher's disease develop pathological features also found in GCL and Sandhoff disease. Therefore, it was reasonable to speculate that conserved cell death pathways might be at play in all three disorders. Preliminary data from Vitner et al. argued in favour of Ripk1 and Ripk3 overrepresentation in the brain of a GCL animal model of disease, the twitcher mouse.

We have crossed the twitcher (*Galc*) and Sandhoff (*Hexb*) mouse strains with the Ripk3 knock-out to homozygosity to test the hypothesis that Ripk3 deficiency might improve disease outcome. We now report that survival was not improved – humane end point mean \pm SD in days was: *Galc*^{-/-}*Ripk3*^{-/-} (n=22) 38.7 \pm 1.3, *Galc*^{-/-}*Ripk3*^{+/-} or *Galc*^{-/-}*Ripk3*^{+/+} (n=25) 37.9 \pm 3.3, *Hexb*^{-/-}*Ripk3*^{-/-} (n=15) 119.6 \pm 3.6 and *Hexb*^{-/-}*Ripk3*^{+/-} or *Hexb*^{-/-}*Ripk3*^{+/+} (n=19) 119.7 \pm 1.7. Neither were disease signs delayed nor hallmark pathological features modified. We conclude that Ripk3 deficiency on its own does not favourably impact the normal course of disease progression in these authentic animal models of disease.

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Preferred presentation
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Gaucher disease: a new silent mutation causing the complete exon 4 skipping of GBA gene

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Background: Gaucher disease (GD) is a recessive inherited disorder of sphingolipid metabolism caused by deficiency of the β -glucosidase enzyme (GBA). GD presents with different clinical phenotypes resulting in a broad spectrum of disease manifestations including hepatosplenomegaly, anemia, thrombocytopenia, bone disease and, at times, neurological involvement. On the basis of the presence and rate of progression of neurological symptoms, GD has been divided into three forms: type 1 (nonneuronopathic), type 2 (acute neuronopathic), and type 3 (subacute neuronopathic). Diagnosis is performed through biochemical and molecular analysis.

Objective: Singling out disease-causing mutations from harmless nucleotide polymorphisms/substitutions is a critical area of research in human genetic diseases.

Here we aimed to characterise the GBA genetic lesions in a 14 years old girl, with low GBA enzyme activity, bearing apparently only one clear-cut mutation in the GBA gene.

Methods: Enzymatic testing was performed on isolated leukocytes. Molecular analysis of the GBA gene was carried out to confirm the enzymatic deficiency. In silico analysis, RNA extraction, RT-PCR analysis and quantitative Real time-PCR analyses were performed to investigate the role of a new GBA gene synonymous variant.

Results: Molecular analysis of the GBA gene revealed only one clear-cut mutation: the c.680A>G (p.N188S) inherited from the maternal allele.

Bioinformatic analysis, extended to all the nucleotide variants identified, showed that the synonymous change c.363A>G (p.G82G), inherited from her father, leads to the loss of an Exonic Splicing Enhancer (ESE).

This hypothesis was investigated with RNA studies in the patient, her parents and controls.

An aberrant transcript, showing a total skipping of exon 4, identified only in the patient and her father, confirmed the pathogenicity of this new variant: the loss of the ESE, due to the c.363A>G nucleotidic change, causes the total skipping of exon 4 during the splicing of the GBA gene. **Conclusions:**

To date the c.363A>G (p.G82G) is the second synonymous mutation, reported on the GBA gene affecting mRNA splicing. These results demonstrate that we must pay close attention to silent mutations and thoroughly investigate their possible pathogenicity also by RNA studies.

It is important to identify which nucleotide changes represent benign polymorphisms and which may result in potential disease-causing mutations so we can make accurate diagnoses, genotype/phenotype correlations, and offer appropriate genetic counselling. To facilitate these distinctions, especially in the case of synonymous changes, the use of in silico prediction methods proved to be very useful.

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Cerebrospinal fluid lysosomal enzymes in Parkinson's Disease

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A prominent feature of Parkinson's disease (PD) pathogenesis is represented by lysosomal dysfunction, which is related to defective alpha-synuclein (α -syn) clearance. Mutations in the GBA gene, encoding for the lysosomal hydrolase β -glucocerebrosidase (GCCase, EC = 3.2.1.45), cause Gaucher disease a rare lysosomal storage disorder, and represent a common risk factor for PD. Patients carrying GBA loss of function mutations have a five-fold increased risk of developing PD with respect to non-carriers and may show a similar phenotype to idiopathic PD. The GCCase substrate, glucosylceramide, stabilizes soluble oligomeric α -syn species. As a consequence, GCCase deficiency may contribute to α -syn aggregation and accumulation. In a recent study, carried out in a PD cohort compared to neurological controls, GCCase activity was found to be reduced in cerebrospinal fluid (CSF) of PD patients, particularly in the earlier stages of the disease, as opposite to an increased oligomeric/total alpha-synuclein ratio. The combination of these measures significantly discriminated PD patients from neurological controls; beta-hexosaminidase was also increased. In another investigation carried out in CSF of PD patients and healthy controls. GCCase activity was found to be slightly but not significantly reduced, together with a significant increase of cathepsin E activity. Lysosomal enzymes were recently assessed in fibroblasts from PD patients with and without GBA1 showing increased beta-hexosaminidase and decreased GCCase activity in PD group. Finally, a reduced GCCase activity has been confirmed in PD brains, both from cases with and without GBA mutations.

Importantly, an accurate validation study on the pre-analytical factors influencing the measurement in CSF of lysosomal enzymes activity has been recently carried out. Several pre-analytical factors that may influence the activity of these enzymes have been analyzed. The conclusion of the study was that after applying the correct pre-analytical operating procedures, the measurement of these enzymes can be considered reliable and reproducible. In the present study the activity of beta-glucocerebrosidase, beta-hexosaminidase, cathepsin D, and cathepsin E were determined in the BioFIND cohort obtained by Michael Fox Foundation. The BioFIND Cohort represents the ideal set for confirming the potential role as PD biomarker of CSF lysosomal enzyme activities. CSF and DNA specimens from 80 PD and 60 controls have been analyzed. The preliminary results confirm a significative reduction of beta-glucocerebrosidase activity in the PD samples in comparison with the control. An increased beta-hexosaminidase and cathepsin E activity was also found in the PD samples in comparison with the control. These results further support the use of beta-glucocerebrosidase activity in the CSF as an early biomarker for Parkinson's disease.

Acknowledgments. This work was supported by Michael Fox Foundation for Parkinson's Disease.

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Lipid-based vectors for RNA interference affect lysosomal function and alter the expression of the lysosomal NPC1 protein

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RNA interference (RNAi) is a mechanism for specific gene silencing at the post-transcriptional level. Short interfering RNAs (siRNAs) guide the RNA-induced silencing complex (RISC) to complementary target mRNA which is then degraded, suppressing gene expression. Exogenous siRNA provided to the cell will also activate this mechanism, making RNAi a powerful research tool and a potential therapeutic strategy in a number of diseases. Delivery of siRNA to cells remains challenging, as the large, hydrophilic siRNA molecules are unable to diffuse across the plasma membrane.

Lipid-based or liposomal vectors are widely used to deliver siRNA to cells, as well as for gene expression and other methods of genetic modification. These vectors are designed to enter cells via the endocytic system and deliver genetic material to the cytoplasm by disrupting endocytic vesicles. Development of these vectors has focused on manipulating the endocytic system to improve transfection efficiency, and recent studies have shown that altered lysosomal function (for example, in lysosomal storage disorders) can impact on efficacy of gene silencing. However, the mechanisms by which lipid vectors deliver their cargo may also disrupt lysosomal function, causing problems when used to study the lysosome.

A number of lipid-based vectors, used according to manufacturer's protocols, were tested for effects on lysosomal function. Initial screening using a LysoTracker assay identified several vectors that induced lysosomal expansion, which were then selected for further characterisation. These vectors had a range of different effects on lysosomal function, including induction of phospholipidosis and accumulation of other lipids in lysosomes, altered endocytic trafficking, and modulation of NPC1 expression.

This study demonstrates that some lipid vectors independently disrupt lysosomal function, endocytosis, and possibly autophagy, making them unsuitable for use in studies of lysosomal function or lysosomal diseases. Other lipid vectors interact with lysosomal function in a manner that may improve transfection efficiency and gene silencing, or potentially in a way that could directly benefit the cell. In particular, elevation of NPC1 levels by certain lipid vectors could prove to be a useful therapeutic tool. These findings will help to refine and improve the tools used to study lysosomal disease, will help direct development of more effective transfection vectors, and will contribute to the development of suitable methods for therapeutic use of RNAi, particularly for lysosomal diseases.

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Preferred presentation

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Lysosomal involvement in the pathogenesis of Huntington's disease

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Huntington's disease (HD) is a fatal inherited neurodegenerative disorder caused by expansion of a polyglutamine tract in the huntingtin protein, and characterised clinically by motor, cognitive and psychiatric deficits. The cellular pathology, and the mechanisms by which polyglutamine expansion in huntingtin leads to disease, are complex and poorly understood.

We have identified a defect in trafficking and function of the lysosomal transmembrane protein NPC1 in HD. This protein plays a role in endocytic lipid trafficking and Ca²⁺ homeostasis, and mutations in this protein lead to Niemann-Pick type C disease (NPC), a rare and fatal neurodegenerative disease characterised by lysosomal storage of lipids. Mislocalisation of NPC1 and a deficit in lysosomal function are observed in HD, potentially due to a direct interaction between NPC1 and huntingtin.

The existence of this protein defect in HD is supported by the presence of lysosomal dysfunction matching that seen in NPC. Storage of characteristic lipids including cholesterol and sphingolipids, lysosomal Ca²⁺ signalling defects, and a block in endocytic trafficking and autophagic vacuole clearance are present in multiple models of HD.

One licensed disease-modifying therapy is currently available for NPC, the glycosphingolipid-biosynthesis inhibitor miglustat. This drug partially restores lysosomal Ca²⁺ signalling and endocytic trafficking and slows clinical progression in NPC. Treatment of HD cellular models, including patient iPSC-derived neurons, with miglustat reduced lysosomal defects, improved cellular trafficking, and was protective against excitotoxic cell death.

This novel finding contributes to understanding of HD pathogenesis, and may help to unravel the mixed reports of altered lipid homeostasis in HD. It may also suggest novel transport pathways by which NPC1 is trafficked to the lysosome. Correct lysosomal function is vital to the cell, and the defects we have observed may explain some of the known problems in Huntington's disease, for example in cellular trafficking and Ca²⁺ homeostasis. Our work has also highlighted an approved small molecule as a novel therapeutic strategy for HD.

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Preferred presentation

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Tangier Disease and Niemann-Pick type C disease: mechanistic convergence and shared therapeutic targets

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Tangier disease is caused by a mutation in the ATP binding cassette transporter A1 (ABCA1), which results in abnormal sterol levels and HDL formation. It has been proposed that ABCA1 is responsible for the transport of lipids and sterols between late endocytic vesicles and apolipoprotein A1 (apoA1), playing a role in HDL metabolism. A mutation in the ABCA1 protein, as seen in Tangier disease cells, results in defective late endocytic vesicular transport and cholesterol accumulation. Currently, there is no specific therapy for Tangier disease. Following the misdiagnosis of a Tangier patient with Niemann-Pick disease type C (NPC) and subsequent clinical improvement on miglustat, we investigated whether these diseases, share convergent pathogenic cell biological features. Tangier disease cells were found to have multiple shared phenotypes with NPC disease, including an enlarged acidic compartment, disrupted endocytic transport of glycosphingolipids, and cholesterol accumulation. Tangier disease cells were also found to have defects in acidic store calcium levels, a disease characteristic found to occur in NPC disease – suggesting a link specifically between Tangier and NPC disease rather than lysosomal storage disorders in general. Additionally, following miglustat treatment both the acidic store calcium defect and glycosphingolipid levels were rescued. Taken together, these data suggest a potential mechanistic links between Tangier and NPC and that miglustat may be an unanticipated therapy for Tangier disease.

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Defining spinal cord neuropathology in a mouse model of Infantile Neuronal Ceroid Lipofuscinosis (INCL) and assessing the efficacy of intrathecal Enzyme Replacement Therapy (ERT).

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The neuronal ceroid lipofuscinoses (NCLs) are a group of recessively inherited, progressive childhood neurodegenerative lysosomal storage disorders. Each is caused by a mutation in a different gene and all forms are fatal. Infantile NCL (INCL, CLN1 disease) is caused by mutations in the CLN1 gene, which codes for the lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1) and is the most rapidly progressing form of NCL. The creation of Ppt1 null mutant mice (Ppt1^{-/-}) has helped to characterize neuropathology within the forebrain, but little is known of the pathology in the spinal cord. Furthermore, while various forebrain-directed therapeutic strategies have been tested pre-clinically, for these to be effective, they will need to be targeted to all affected brain regions.

Using immunohistochemical and stereological techniques, our analysis has revealed there to be pronounced neurodegenerative and neuroinflammatory changes in the spinal cord of INCL mice. These changes occur at all levels of the cord and worsen with disease progression. We have documented progressive increases in storage burden, differences in gray and white matter volume, loss of dorsal and ventral horn neurons and in astrogliosis and microglial activation. None of these pathologies had been impacted in INCL mice that receive forebrain targeted gene transfer, but our novel data from the spinal cord suggest it to be an important therapeutic target. To begin testing such approaches we have assessed the impact of delivering enzyme replacement therapy (ERT) intrathecally to INCL mice, which seems to partly alleviate the pathological phenotypes we have documented in the spinal cord. This work is significant in revealing the presence of pronounced spinal cord pathology, but has important therapeutic implications for this and other forms of NCL.

Preferred presentation

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Pld3 – A new lysosomal protein implicated in Alzheimer`s disease

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Alzheimer`s disease is the most common neurodegenerative disease of aging and clinically heterogeneous with a strong genetic component. Even though the precise pathomechanisms ultimately leading to neuronal cell death still remain elusive, it is widely accepted that toxic amyloid beta fragments derived from the amyloid precursor protein (APP) contribute to the pathology by forming plaques. However, in the past a number of biological processes have been implicated in the pathogenesis by influencing generation of amyloid beta including autophagy and lysosomal degradation pathways. Of note, lysosomal alterations are seen early in AD and even precede the formation of amyloid plaques (1).

Several genes have been associated with AD risk in the last 20 years. Very recently, a new risk gene was identified by whole-exome sequencing in late-onset Alzheimer`s disease families (2). In this study, Pld3, coding for a putative phospholipase D, was shown to double the risk to develop the disease (2). Pld3 is a predicted type II transmembrane protein with so far unknown function previously assigned to the endoplasmic reticulum (3). Its expression is highest in the brain.

We provide clear data that Pld3 is a new resident lysosomal protein. Pld3 co-localizes both in overexpressing cells and on the endogenous level with lysosomal markers. After reaching late endosomes and lysosomes as a transmembrane protein via the plasma membrane, Pld3 is cleaved to a soluble and stable luminal domain and a short membrane-bound N-terminal fragment which is degraded rapidly. Pld3 deficient mice show a mild phenotype with localized mild microgliosis restricted to the dentate gyrus and the corpus callosum. Whereas APP levels are unaffected in brains of Pld3 deficient mice, Pld3 co-localizes with APP in endosomes and Pld3-overexpression in cultivated cells leads to significant decrease in the levels of full length APP and APP fragments.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

TDP-43 pathology in Niemann pick type C

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Niemann Pick C disease (NPCD) is a lysosomal storage disorder due to the deficient function of NPC1 or NPC2, two proteins involved in the intracellular transport of unesterified cholesterol. NPCD is characterized by the occurrence of visceral and neurological symptoms. At present, the molecular mechanisms that link this disease with the progressive neurodegeneration observed in patients are not fully elucidated and the therapeutic options are limited.

TDP-43 (TAR DNA-binding protein 43) is a main component of brain inclusions in amyotrophic lateral sclerosis frontotemporal lobar degeneration patients, and it plays a major role in neurodegenerative processes probably by multiple mechanisms that include both gain and loss of function. Therefore, we investigated whether TDP-43 pathology is present in NPCD. We first studied the expression of TDP-43 in the cerebellum of wild-type and *Npc1*^{-/-} mouse. In this model, TDP-43 protein levels were significantly reduced at post-natal day 11, when neuropathological signs of the disease have not yet appeared, and they progressively decrease until day 75 when the protein was almost undetectable. To extend these observations to the human disease, we then analyzed the expression and intracellular localization of TDP-43 in a human neuronal model of NPC D obtained through the differentiation of multipotent adult stem cells (MASCs) isolated from patients affected by NPCD disease. In this human neuronal model of the disease, the levels TDP-43 mRNA and protein were not significantly different from those detected in cells derived from controls. However, while in control cells TDP-43 was mostly confined within the cell nuclei, as previously reported, it was mainly localized in the cytoplasm and associated with markers of stress granules in most NPC derived cells. Furthermore, in NPC cells TDP-43 mislocalization was associated with hyperphosphorylation. In order to investigate the possible functional impact of TDP-43 mislocalization, we have then analyzed the mRNA expression of a set of genes known to be regulated by TDP-43 and involved in neuronal survival and differentiation Six genes (FAP2A, CNTFR, MAF2D, MADD, TLE1 e TNIK) were differentially expressed in cells derived from NPC patients with respect to healthy controls. Interestingly, treatment of NPC cells with N-Acetyl-cystein (NAC), a well known anti-oxidant agent or with beta-cyclodextrin, a cholesterol sequestering agent that mobilise cholesterol from the late endosomal/lysosomal compartment, partially restored both TDP-43 localization and TNIK e TFAP2A expression levels.

These results suggest that in NPCD, cholesterol storage leads to a chronic stress condition that triggers the translocation of TDP-43 from the nucleus to the cytosol where it is phosphorylated and recruited within stress granules. As a consequence, its nuclear function might be impaired.

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Novel approaches to rescue normal splicing of GAA transcripts carrying the common c.-32-13T>G mutation.

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Glycogen storage disease type II (GSDII), is a lysosomal storage disorder due to mutations of the GAA gene, which causes lysosomal α -glucosidase (GAA) deficiency. Clinically, GSDII has been classified in infantile and late-onset (LO) forms. Most LO patients shared the leaky splicing mutation c.-32-13T>G.

In a previous study we have demonstrated that this mutation abrogates the binding of the splicing factor U2AF65 to the polypyrimidine tract of exon 2 of GAA, affecting the overall splicing efficiency and leading to the partial or total exclusion of exon 2 from the mature transcript. In addition, we have shown that it is possible to partially rescue normal splicing of c.-32-13T>G mutant transcripts by over-expressing the splicing factor SF5R4 or by the action of small molecules.

In this study, we have explored two different strategies aimed promote inclusion of the full exon 2 in the final GAA mRNA transcript: 1) the use of modified U1snRNP molecules that target the donor site of exon 2. This strategy is based on the possibility to generate U1snRNP molecules that specifically recognize suboptimal splice sites (as is the case of GAA exon 2) and improve their recognition by the splicing machinery 2) the identification of splicing silencer sequences within the exon 2 of GAA in order to design antisense oligonucleotides which would block the interaction of negative regulators and favour exon inclusion.

Four modified U1 snRNAs that interact with sequences upstream (-3U1 and -7U1) and downstream (+1U1 and +6U1) of the exon 5' splice site by complementarity, have been tested in vitro in HeLa cells transfected with a minigene containing entire exon 2 and 50 nt of the downstream and upstream intronic regions (in its wild type and mutant version), inserted in the pTB plasmid. The effect on exon 2 inclusion was tested by RT-PCR using minigene-specific primers. In all cases a significant increase of exon 2 inclusion of the mutant minigene was observed.

On the other hand, with the aim to identified possible silencer sequences within the intron 2, the mutant minigene has been used to build 7 deletion construct in which overlapping regions of 140 bp each were eliminated from the original construct. The deleted minigenes were transfected into HeLa cells and the effect of each deletion on exon 2 inclusion was tested by RT-PCR. The deletion of a region located between 217 and 356 nt downstream of the 3' splice site of exon 2 resulted in a significant increase of exon 2 inclusion, suggesting the presence of silencer sequences within this region.

In conclusion, we have demonstrated that modified U1 snRNAs may constitute a promising therapeutic strategy to rescue normal splicing of c.-32-13T>G mutant alleles. In addition, we have identified splicing silencer sequences within exon 2 of GAA gene which could represent possible targets for the development of antisense oligonucleotides based therapies.

Preferred presentation

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Search for genetic and epigenetic determinants in MPS VI clinical phenotype: NGS analysis of two monozygotic twins

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Maroteaux-Lamy Syndrome (Mucopolysaccharidosis type VI, MPSVI) is an inherited autosomal recessive lysosomal storage disorder due to the deficit of arylsulfatase B enzyme. The deficit leads to a progressive multisystemic disease with high genetic and phenotypic heterogeneity, often making genotype-phenotype correlation unfeasible. In this study we evaluated a female MPSVI monozygotic twins couple, carrying the mutation p.L321P in homozygosis and showing at start of ERT some discordant signs and symptoms. A series of NGS analyses was carried out with the main goal to highlight possible genetic and epigenetic factors able to explain the clinical differences between the twins.

A low coverage genome sequencing was performed on leucocytes, confirming twins' monozygosity and evidencing no structural variants. Exome sequencing on both blood and skin DNA revealed several somatic variants which were filtered by the in-house developed QueryOR platform [1], using known associations between genes and phenotypic traits available from different databases (DisGeNET, HPO, GWAS Central and GWAS Catalog). Overall, 41 genes with different variants in the 2 twins were selected as possibly causative for the discordant traits between the twins.

RNA-seq on skin fibroblasts RNA, evidenced 164 differentially expressed genes, which did not highlight significantly enriched Gene Ontology terms, while the filtration for association with the discordant phenotypic traits, resulted in 23 genes. Among them, MMP1 and MMP3 could be considered interesting as some SNPs in their promoters have been associated with mitral valve regurgitation severity [2] and with scoliosis [3] which are two traits discordant between the twins.

Methylome sequencing performed in both tissues, allowed the analysis of over 3.7M CpG dinucleotides. A methylation score was calculated for each gene, considering the proximity to differentially methylated regions and the entity of methylation. The results indicate a good correlation between methylation and gene expression.

Being MPS VI so rare, the chance to study 2 affected twins with discordant phenotype represents a unique opportunity. The analysis, beyond the results above mentioned, has also contributed to define a general pipeline to address the open question of genotype-phenotype correlation in these diseases.

The study was partly funded by Biomarin Pharmaceutical Inc. and by University of Padova within the BIOINFOGEN Strategic Project frame.

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Preferred presentation
I only want to present a POSTER

Inherited Neurometabolic Diseases Database from InNerMeD-I-network EU project

D'Avanzo F¹, Bellettato CM¹, Bartoloni F², Barić I³, Garcia Cazorla A⁴, i Dali C⁵, Ceci A², Scarpa M¹ on behalf of all InNerMeD partners

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Inherited NeuroMetabolic Disorders (iNMDs) are a group of rare genetic metabolic diseases that affect the brain causing mental retardation and progressive neurodegeneration and that, if not promptly treated, could end in early death. Lack of information on these conditions can lead to delayed diagnosis and treatment, with consequent tragic results. Increasing awareness is therefore the first crucial step in fighting these pathologies.

In this regard, the Directorate-General for Health & Consumers of the European Commission has approved the project "Inherited NeuRoMetabolic Diseases Information Network" (InNerMeD-I-network), aiming to create a network of information targeted on iNMDs.

One of the most important product of InNerMeD project will be the release of an electronic repository containing information on research, diagnosis and treatment of iNMDs.

Considering that a universally accepted definition as well as a complete list and a classification of iNMDs do not actually exist, the first step in this task has been the production of a definition and a classification of iNMDs. InNerMeD partners agreed with the following definition: "Genetic metabolic disorders showing clinical neurologic/cognitive symptoms at any time during the disease progression".

At first, a list and a classification of inherited metabolic diseases has been produced starting from different sources [1-4], then for each disease an examination of the neurological involvement has been performed by a group of experts. For the resulting list of iNMDs, scientific validated information are being retrieved after the definition of procedures and methodology for bibliographic searching and data collection. The repository will contain data on: diseases (clinical information, laboratory and instrumental examinations, diagnosis, prevention, guidelines), therapies (medicinal products and therapies under research, with related details including EMA/FDA orphan drugs) and centres of expertise treating the diseases.

Being the project's target audience very various, a dictionary of medical terms [5] has been linked to the texts of the repository in order to facilitate the consultation by non-scientific users.

The repository is accessible from the InNerMeD project website (www.innermed.eu).

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Preferred presentation

I only want to present a POSTER

Subunit interactions of mucopolipidosis II and III-related hexameric GlcNAc-1-phosphotransferase complex

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Mucopolipidoses II and III are inherited lysosomal storage disorders which are caused by defects in the Golgi-resident GlcNAc-1-phosphotransferase. The GlcNAc-1-phosphotransferase is a hexameric complex consisting of two alpha-, two beta- and two gamma-subunits that catalyzes the formation of mannose 6-phosphate (M6P) recognition marker on lysosomal enzymes, required for their efficient receptor-mediated transport to lysosomes. GNPTAB and GNPTG encode the alpha/beta-subunit precursor membrane protein and the soluble gamma-subunit, respectively. The cleavage of the alpha/beta-subunit precursor to mature alpha- and beta-subunits by the site-1 protease in the Golgi lumen is essential for GlcNAc-1-phosphotransferase activity. Performing extensive mutational analysis, we identified the binding region of the gamma-subunit in a previously uncharacterized domain of the alpha-subunit, afterward named GNPTG binding (GB) domain. Both deletion of GB preventing gamma-subunit binding and CRISPR/Cas9-targeted deletion of GNPTG led to significant reduction of GlcNAc-1-phosphotransferase activity. We also identified the cysteine residue responsible for covalent homodimerization of alpha-subunits. Homodimer formation of alpha-subunits is, however, neither required for interaction with gamma-subunits nor for catalytic activity of the enzyme complex. Finally, binding assays using various gamma-subunit mutants revealed that residues in the M6P receptor homology (MRH) domain of gamma-subunits are responsible for the interaction with glycosylated alpha-subunits. These studies provide new insight into the assembly of the GlcNAc-1-phosphotransferase complex, and the functions of distinct domains of the alpha- and gamma-subunits, and might improve predictions of the clinical course of MLII/MLIII based on the genotype.

Preferred presentation

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Cardiac disease in the murine model of mucopolysaccharidosis IIIB

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Mucopolysaccharidosis (MPS) IIIB is a lysosomal disease due to the deficiency of the enzyme α -N-acetylglucosaminidase (NAGLU) required for heparan sulfate (HS) degradation. The disease is characterized by mild somatic features and severe neurological disorders. Very little is known on the cardiac dysfunctions in MPS IIIB (1). In this study, we used the murine model of MPS IIIB (NAGLU knockout mice, NAGLU^{-/-}) (2) in order to investigate the cardiac involvement in the disease. Echocardiographic analysis showed a marked increase in left ventricular (LV) mass, reduced cardiac function and valvular defects in NAGLU^{-/-} mice as compared to wild-type (WT) littermates. The NAGLU^{-/-} mice exhibited a significant increase in aortic and mitral annulus dimension with a progressive elongation and thickening of anterior mitral valve leaflet. A severe mitral regurgitation with reduction in mitral inflow E-wave-to-A-wave ratio was observed in 32-week-old NAGLU^{-/-} mice. Compared to WT mice, NAGLU^{-/-} mice exhibited a significantly lower survival with increased mortality observed in particular after 25 weeks of age. Histopathological analysis revealed a significant increase of myocardial fiber vacuolization, accumulation of HS in the myocardial vacuoles, recruitment of inflammatory cells and collagen deposition within the myocardium, and an increase of LV fibrosis in NAGLU^{-/-} mice compared to WT mice. Biochemical analysis of heart samples from affected mice showed increased expression levels of cardiac failure hallmarks such as calcium/calmodulin-dependent protein kinase II, connexin43, α -smooth muscle actin, α -actinin, atrial and brain natriuretic peptides, and myosin heavy polypeptide 7. Furthermore, heart samples from NAGLU^{-/-} mice showed enhanced expression of the lysosome-associated membrane protein-2 (LAMP2), and the autophagic markers Beclin1 and LC3 isoform II (LC3-II). Overall, our findings demonstrate that NAGLU^{-/-} mice develop heart disease, valvular abnormalities and cardiac failure associated with an impaired lysosomal autophagic flux.

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Preferred presentation
I only want to present a POSTER

MPS II in the three-year-old girl. Biochemical and molecular findings and cell biology study, Generation of iPSC model

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Mucopolysaccharidosis type II (Hunter syndrome, MPS II, OMIM 309900) is an X-linked recessively inherited lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase activity (IDS, EC 3.1.6.13). The majority of patients are males and heterozygotes usually do not show any symptoms of the disease. On the other hand at least 16 female patients with signs of MPS II due to IDS deficiency have been reported. We present a 3-year old girl with coarse face, mild hepatosplenomegaly, dysostosis multiplex and mild psychomotor retardation. Increased urinary glycosaminoglycans (GAGs) and electrophoretic pattern of GAGs as seen in patients with MPS I, VI, VII and male MPS II patients were found. Activity of α -iduronidase and β -glucuronidase in leukocytes was in the range of control. Decreased IDS activity with values comparable to male patients with MPS II in both serum and leukocytes was revealed. Activities of other sulphatases were normal thus excluding multiple sulphatase deficiency.

In addition, the diagnosis of MPS II was established at the molecular level. In the IDS gene, a heterozygous substitution c.1403G>A (p.Arg468Gln), which is a common mutation in MPS II patients, was found as a de novo mutation. No other mutation which could explain the manifestation of MPS II in the girl was found. The analyses of the patient's cDNA showed that only the mutated allele was transcribed. Determination of X-inactivation status showed preferential inactivation of maternal allele (96/4 to 99/1) in patient leukocytes and buccal swabs. This finding is in accordance with the published data which demonstrate that extremely skewed X-inactivation leading to almost exclusive expression of the mutated allele is a common feature in female patients manifesting MPS II.

For further studies, leukocytes derived iPSC cell lines were established and deficiency of IDS activity and mutation in the IDS gene were confirmed. X-inactivation analysis showed almost completely skewed X-inactivation with only mutant allele active. These data can be explained by clonal origin of the iPSC lines.

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Preferred presentation
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Crispr/Cas9 Generation Of Ipsc Model Of Fabry And Schindler Disease

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Human pluripotent stem cells (iPSc) generated from various cell types of patients offer a unique opportunity to produce disease-relevant cell types for pathophysiology studies and development of new therapeutic approaches as they possess unlimited self-renewal capacity and potential to generate all adult cell types, including rare or inaccessible human cell population. However, due to rarity of many genetic disorders, the opportunity to obtain biological samples is often exclusive.

Recently, an efficient genome manipulation technology using the RNA-guided DNase Cas9, the clustered regularly interspaced short palindromic repeats (CRISPR) system, has become widely used for rapid and highly efficient generation of knockout human iPSC used in modeling of disease conditions. CRISPR/Cas9 system induce double strand breaks (DSBs) at desired genomic loci, triggering the endogenous DNA repair machinery. Processing of DSBs by the error-prone nonhomologous end-joining (NHES) pathway leads to small insertions and deletions (indels) useful for generating loss-of-function mutations.

In our study, knockouts in two lysosomal hydrolases, alpha-galactosidase A gene (GLA, Fabry disease) and alpha-N-acetylgalactosaminidase gene (NAGA, Schindler disease) were made by this technology. Moreover, to help elucidate biological significance of substrate specificity overlap of GLA and NAGA, double knockouts of both genes were produces. The successful generation of the models were confirmed by demonstration of deficient activities in the iPSC lines and sequencing of the GLA and NAGA genes. The cells were used for initial experiments including study of natural substrates degradation (B-6-2, Gb3Cer, A-6-2) in GLA, NAGA and double knockout differentiated cells.

Preferred presentation
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Pre-clinical workup of lentiviral mediated stem cell gene therapy for Mucopolysaccharidosis type IIIA

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Sanfilippo disease (MPSIIIA) is a devastating lysosomal storage disease (LSD) that causes progressive mental decline leading to death in early childhood. Absence of functional N-sulphoglucosamine sulphohydrolase (SGSH) leads to accumulation of Heparan Sulphate (HS) in the lysosomes of patients, causing cellular dysfunction, with the brain predominantly affected. There are currently no treatments. Enzyme replacement therapy is ineffective since enzyme cannot pass the blood brain barrier. Haematopoietic stem cell transplant (HSCT) can circumvent this problem via monocyte trafficking and engraftment in the brain, thereby allowing delivery of enzyme by cross correction. HSCT corrects the brain in the related HS storage disease MPSI Hurler, however this is not the case for MPSIIIA due to insufficient enzyme production. To overcome this we developed a lentiviral vector (LV) mediated stem cell gene therapy approach to significantly boost SGSH enzyme levels. Eleven percent of normal enzyme activity was achieved in the brain which corrected the behavioural phenotype in MPSIIIA mice, normalised GAG storage and neuroinflammation, corrected lysosomal compartment size and significantly improved survival.

Pre-clinical toxicology studies have demonstrated safety and efficacy of GMP grade SGSH LV in human CD34+ cells. We have achieved between 2 and 4 vector copies in transduced human HSCs, resulting in an increase in SGSH enzyme activity of approximately 10 -20 fold normal levels without any adverse toxicity or lineage skewing. Furthermore, we observed low transformation frequency using the in vitro immortalisation assay, comparable to other lentiviral vectors currently in the clinic. We aim to begin a phase I/II clinical trial in MPSIIIA patients in 2016.

Preferred presentation

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LAMP1 and LAMP2 influence the intracellular lipid homeostasis

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We have shown that cholesterol efflux from late endosomes/lysosomes (LE/LY) is defective in LAMP2-deficient cells, and that LAMP2-deficient mice have elevated plasma LDL profiles (1), suggesting a role for LAMP2 in cholesterol efflux from LE/LY and in LDL cholesterol clearance. We have characterized the regions in LAMP2 molecule that are critical for its function in cholesterol efflux and shown that the luminal domain, in particular its membrane proximal region and one of the surface-exposed loops, as well as the transmembrane region, are needed for efflux of cholesterol from LE/LY. Using microscopic methods and lipid mass spectrometry, we have characterized the lipids in wild type and LAMP1/LAMP2 double deficient (LAMP^{-/-}) cell lines. In addition to free cholesterol, the LAMP^{-/-} cells accumulate the lysosomal lipid bis(monoacylglycerol) phosphate (BMP) and phosphatidic acid. The accumulation of cholesterol and BMP in LAMP^{-/-} cells increased with increasing passage number, which may be connected with the simultaneous decrease in LAMP2 protein levels. Electron microscopy showed that free cholesterol and BMP accumulated both in the limiting and internal membranes in LAMP^{-/-} LE/LY. Lipid mass spectrometry also detected changes in the fatty acid composition of several lipid species in LAMP^{-/-} cells. Taken together, our results suggest that LAMP1 and in particular LAMP2 participate in the efflux of lipids from LE/LY and in intracellular lipid homeostasis.

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Preferred presentation
I have no preference

Combination of low-dose gene therapy and monthly enzyme replacement therapy improves the phenotype of a mouse model of lysosomal storage disease

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Enzyme replacement therapy (ERT) is the standard of care for several lysosomal storage diseases (LSDs). However, ERT requires multiple and costly administrations and has limited efficacy on several LSDs features¹. We have recently shown that a single administration of 2x10¹² gc/kg of adeno-associated viral vector serotype 8 (AAV2/8) is at least as effective as weekly ERT in MPS VI mice². However, the administration of high doses of AAV2/8, which require a challenging and costly production process, might result in cell-mediated immune responses to AAV³. Here we evaluated whether the combination of low doses of AAV2/8 (<2x10¹² gc/kg) with a rarified ERT schedule (monthly) may be as effective as the single treatments at high doses or frequent regimen. While normalization of glycosaminoglycans (GAG) levels was observed in visceral organs regardless of treatment, greater reduction of urinary GAGs was observed in mice receiving the combined therapy. In particular, urinary GAG reduction was comparable to that obtained following either high doses of AAV2/8 or weekly ERT in mice receiving both 6x10¹¹ gc/kg of AAV2/8 and ERT. Similarly, reduction of lysosomal storage in both heart valves and myocardium was more consistent in mice receiving 6x10¹¹ gc/kg of vector, particularly in combination with ERT. These data support a similar efficacy between low-dose gene therapy combined with rarified ERT and the corresponding single treatments at high doses or frequent regimen. This should increase the safety and reduce the risks and costs associated with both therapeutic approaches.

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Preferred presentation
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The challenge of significance of new GLA gene variants: the importance of functional studies

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Background: GLA gene mutations cause alpha-galactosidase A (α -Gal A) enzyme deficiency which leads to the X-linked lysosomal storage disorder Fabry disease (FD). FD is characterized by a heterogeneous spectrum of clinical manifestations and the correlation between new identified GLA nucleotide variants and patients' phenotype represents a challenge. Molecular heterogeneity is an important feature of α -Gal A protein which counts 429 AA and almost 800 known variants so far. Several polymorphic variants and one missense change which causes pseudodeficiency have also been described. Some GLA missense variants, previously considered to be disease-causing on the basis of clinical symptoms and routine sequencing analyses, have recently been reclassified as polymorphisms, due to functional and population studies. Hence, the identification of new GLA variants does not always guarantee the molecular confirmation of FD, particularly in mildly affected patients or in female probands. Functional studies are necessary to shed light on their clinical significance and eventual responsiveness to chemical chaperone treatment (e.g. DGJ).

Aim: to confirm that new GLA gene variants, which we detected in six patients with suspected FD, affect α -Gal A enzyme activity and/or protein by using in vitro expression studies and by evaluating the responsiveness to DGJ.

Results: We identified six new GLA gene variants in six probands (4 males and 2 females): c.159C>G p.(Asn53Lys), c.400T>C p.(Tyr134His), c.680G>C (p.Arg227Pro), c.815A>T p.(Asn272Ile), c.907A>T p.(Ile303Phe) and c.1163_1165delTCC (p.Leu388del). We performed bioinformatic analysis, site-directed mutagenesis and in vitro expression studies in COS-1 cells. Transfected cells were analyzed by α -GAL A enzyme assay and western blot analysis. Reduced α -GAL A activity was detected in all the analyzed mutant constructs. Mutant constructs, expressed in presence of DGJ, revealed a positive response to DGJ treatment only for p.(Asn53Lys) and p.(Ile303Phe) variants, while the other changes were not responsive.

Conclusion: Functional studies carried out on the α -Gal A mutant proteins support a disease-causing effect of the new identified six GLA variants. Two of them are eligible candidate for the treatment with DGJ. Functional studies should be mandatory in presence of a new GLA gene variant, particularly in light of emerging newborn screening programs for lysosomal storage disorders.

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Hsp70-based therapies as clinical candidates for lysosomal storage diseases

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Lysosomal storage diseases (LSDs) often manifest with severe systemic and central nervous system (CNS) symptoms. The existing treatment options are sparse and none of them are effective against the devastating neurological manifestations. We have demonstrated proof-of-concept for Heat shock protein 70 (Hsp70)-based strategies as potential pan-LSD therapies. Recombinant Hsp70 improves the binding of several sphingolipid-degrading enzymes to their essential co-factor, bis(monoacylglycero) phosphate, in vitro and reverts lysosomal pathology in primary fibroblasts from 14 patients with eight different LSDs. It penetrates effectively to murine tissues including CNS, inhibits glycosphingolipid accumulation in murine models of Fabry (GLA^{-/-}), Sandhoff (HEXB^{-/-}) and Niemann-Pick type C (NPC1^{-/-}) diseases, and effectively alleviates a wide spectrum of disease-associated neurological symptoms in HEXB^{-/-} and NPC1^{-/-} mice. Importantly, oral administration of arimoclomol, a clinically enabled small molecule co-inducer of Hsp70, recapitulates the pan-LSD and neurological potential of recombinant Hsp70 encouraging the development of Hsp70-based therapies for LSDs.

Preferred presentation
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AAV-mediated TFEB overexpression alleviates skeletal muscle pathology and motor impairment in a Pompe disease mouse model

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The recent discovery of transcription factor EB (TFEB) role in stimulating autophagy, enhancing lysosomal exocytosis and promoting cellular clearance has prompted the evaluation of TFEB as a therapeutic target for the treatment of several lysosomal storage disorders. Preliminary studies have been performed in cellular and animal models of multiple sulfatase deficiency, mucopolysaccharidosis IIIA, and Pompe disease (PD). PD is a severe metabolic myopathy, due to acid alpha-glucosidase (GAA) deficiency and characterized by both glycogen accumulation into lysosomes and impairment/dysfunction of autophagy. Enzyme replacement therapy (ERT) with human recombinant GAA is currently the only treatment approved for PD; however, ERT shows several limitations and in some patients fails to correct skeletal muscle pathology. We previously showed that intramuscular injection of an AAV-TFEB vector in a PD mouse model resulted in extensive glycogen clearance in injected muscles.

Here we evaluated the effects of systemic TFEB overexpression, through an AAV-mediated approach, on phenotypical rescue in the mouse model of PD. We treated 18 1-month-old PD mice with 1,5x10¹⁴vp/Kg AAV2.9-TFEB by retro-orbital vein injection. A group of animals (n=6) was sacrificed at 3 months for analysis of pathology (glycogen content, PAS staining, Electron Microscopy-EM). A second cohort of animals, treated under the same conditions, was followed at 3, 5 and 8 months for functional analysis.

We observed a reduction in glycogen content by PAS staining in individual muscle fibers of TFEB treated mice. EM showed reduced number (2,42±1,86 vs 3,73±1,35, TFEB-treated mice vs PD untreated mice) and density (127,97±38,11 vs 68,68±19,82, TFEB-treated mice vs PD untreated mice, expressed as translucency) of lysosomes, and reduced number of autophagosomes in TFEB-treated animals, supporting the role of TFEB in autophagosome processing. Behavioral analysis of TFEB-treated animals at different time-points showed significantly improved Rotarod performance compared to untreated animals, suggesting a delay in disease progression.

These results suggest a potential of systemic TFEB overexpression as a therapeutic approach for the treatment of PD.

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Preferred presentation
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Effects of cuprizone induced demyelination in a mouse model of Metachromatic Leukodystrophy

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Introduction: Metachromatic Leukodystrophy (MLD) is caused by deficiency of lysosomal enzyme arylsulfatase A (ASA). ASA catalyzes the degradation of sulfatide, a major myelin component, to galactosylceramide. In case of ASA deficiency, sulfatide accumulates in the lysosome. Patients suffer from severe demyelination and neurodegeneration, rapidly leading to death.

ASA-knockout [ASA(ko)] mice previously have been generated by disruption of the ASA gene. Although ASA(ko) mice show sulfatide storage, myelin remains intact, unlike in human patients. Therefore an enhanced transgenic mouse model [tgASA(ko)] has been generated. Due to the overexpression of galactose-3-O-sulfotransferase-1, tgASA(ko) mice synthesize an increased amount of sulfatide, show increased sulfatide storage and demyelination with ongoing age.

Reasons for demyelination in MLD are still unknown. Physiologically a demyelinating event is frequently followed by endogenous remyelination. A general hypothesis states, that demyelination occurs, when the natural equilibrium between de- and remyelination is disturbed. Therefore we examined, whether demyelination in MLD is caused by impairment of remyelination.

Methods: Feeding bis(cyclohexanone)oxaldihydrazone (cuprizone) to mice is a well-established method to induce demyelination. Subsequent feeding of standard diet allows remyelination to occur.

To induce demyelination, we fed seven week old ASA(ko) mice with 0.2% cuprizone (CPZ) for a period of six weeks, followed by an equally long remyelination phase.

Results: Independent of genotype, histochemical staining of myelin shows CPZ induced demyelination of corpus callosum in treated mice. Upon demyelination body weight is significantly reduced in ASA(ko) mice, but not in ASA(+/-) control. Alcian Blue staining reveals increased sulfatide storage in the brains of CPZ treated ASA(ko), limited to areas of demyelination. Surprisingly, sulfatide levels as determined by TLC remain unchanged. Immunohistochemical stainings reveal that sulfatide storage is restricted to microglia. However, this did not increase cytokine levels in the AS ko mice. Western blot analysis shows significantly reduced levels of myelin basic protein (MBP), proteolipid protein (PLP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in ASA(ko) mice after remyelination phase. Furthermore, myelination related oligodendrocyte lineage factor 2 (OLIG2) is not upregulated during CPZ treatment in ASA(ko).

Conclusion: We show that demyelination increases sulfatide storage in microglia of ASA(ko) mice, accompanied by significant weight loss, strong astrogliosis and significant reduction of myelin related proteins as MBP, CNPase and PLP. In contrast to ASA(+/-) mice, myelin related protein levels of ASA(ko) mice do not recover after six weeks on standard diet and transcription factor Olig2 is not upregulated during CPZ treatment. Our data suggest that an impairment of remyelination may be partly responsible for demyelination in MLD.

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Development of a lentiviral-based gene therapy for Mucopolysaccharidosis II

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Mucopolysaccharidosis type II (MPS II) is an X-linked genetic disorder characterized by mutations in the iduronate-2-sulphatase (IDS) gene, which normally degrades complex sugars in lysosomes. These mutations lead to cellular accumulation of glycosaminoglycans in the brain and skeleton, and culminate by death in teenage years. Severe MPS II is non-responsive to enzyme replacement therapy or standard haematopoietic stem cell (HSC) transplantation. The biggest challenge in developing therapies for MPS is achieving efficient delivery into the central nervous system. We have previously shown that ex vivo gene therapy can correct the MPSIIIA mouse phenotype by overexpressing the missing enzyme and targeting brain cells using a lentivirus. We propose to test HSC gene therapy, by designing third-generation lentiviral vectors driven by a myeloid-specific promoter, hCD11b, to primarily target the brain in MPSII. We have successfully designed and generated high-titre lentiviruses expressing IDS, SUMF1 and TFEB, and are currently evaluating their efficacy in vivo. However, therapeutic efficacy is not only measured biochemically, but also through positive changes in behaviour in the MPSII mouse model. To this effect, we are evaluating the IdS-KO mouse model for neurocognitive, skeletal and activity deficits that are associated with severe MPSII patients. We are assessing the severity and progression of the MPSII phenotype in independent cohorts of male 2-, 4-, 6- and 8-months of age mice through open-field, spontaneous alternation, novel object recognition, rotarod and other skeletal analyses to obtain robust sets of baseline data.

Preferred presentation
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The complex mechanism of therapeutic action of Miglustat in Niemann-Pick type C disease

Haslett LJ, Lloyd-Evans E
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Numerous publications have shown miglustat (N-butyldeoxynojirimycin) to have beneficial effects in NPC patients, mice and cells and at present it is the only therapy approved for the treatment of the neurological manifestations of NPC disease in the EU(1,2,3). Although miglustat is known to be capable of reducing lysosomal glycosphingolipid (GSL) storage it is not completely understood how miglustat is therapeutically active in NPC patients especially as mouse models which cannot synthesise gangliosides do not show the same benefit as NPC mouse models treated with miglustat(4). If we fully understand how miglustat works we may be able to develop an improved therapeutic strategy.

Initially, we measured all of the lipids stored in NPC cells after miglustat treatment and noted that there was not only a reduction in all GSLs but also a reduction in the levels of sphingosine. Further to this we have observed a partial correction of the lysosomal Ca²⁺ deficit alongside improved trafficking of ganglioside GM1. These findings were observed after treatment with both high concentrations of miglustat and a long-term lower concentration treatment designed to mimic patient cerebro-spinal fluid concentration of miglustat(2). This is the first proposed explanation for the clinically beneficial affects of miglustat.

Conversely, however, we did not observe any reduction in other major classes of storage lipid or correction of cellular sphingosine localisation. High levels of cholesterol and lysobisphosphatidic acid were still evident and the levels of sphingomyelin in miglustat treated were markedly increased. This prompted us to look at the enzyme responsible for the breakdown of sphingomyelin; acid sphingomyelinase (ASM). Previous work has shown that in NPC patient sample homogenates ASM activity is not decreased(5) but using immunofluorescent staining we have shown that ASM does not localise to the storage organelle in NPC cells. Treatment with miglustat does not improve this defect in localisation and when coupled to the biosynthetic elevation of sphingomyelin instead of GSLs this ultimately results in reduced cellular breakdown of sphingolipids to sphingosine within lysosomes.

We have identified that reduction in sphingosine is the major therapeutic benefit of miglustat as it can partially correct lysosomal Ca²⁺ defects and subsequently improve endocytic defects such as trafficking of ganglioside GM1 in addition to the expected improvement in GSL storage. Our work suggests that in order to improve the therapeutic benefit of miglustat it may be necessary to improve the concentration of the drug entering the brain. Furthermore, our findings indicate that targeting sphingosine accumulation is a key therapeutic intervention point for NPC disease.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Cellular phenotypes of CLN3 disease present new opportunities for phenotypic screening

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The neuronal ceroid lipofuscinoses (NCLs) are one group of the 70 or so lysosomal storage disorders (LSDs) which are rare inherited diseases. They often result in severe neurodegeneration in the first or second decades of life with ultimately fatal outcomes. One of the most prevalent forms of NCL, juvenile NCL (now mainly referred to as CLN3 disease) is caused by mutations in the CLN3 gene which most often presents with visual impairment between the ages of 4 and 8 followed by epilepsy and progressive neurodegeneration. The mean age of death for CLN3 disease patients is 24 and currently there is no therapy for this disease. The protein product of this gene, CLN3, is an endolysosomal transmembrane protein whose function has yet to be defined(1), lack of information on the function of this protein has hampered therapeutic development. Recently we have measured the cellular Ca²⁺ levels of cerebellar neuronal cells which express the most common disease causing mutation, a 1kb deletion in Cln3. We observed robustly increased levels of Ca²⁺ in the lysosomes of cells expressing mutant Cln3 alongside decreased mitochondrial Ca²⁺ stores and reduced store-operated Ca²⁺ entry. Ca²⁺ homeostasis in the endoplasmic reticulum (ER) of cells expressing mutant Cln3 was also disrupted, although the total Ca²⁺ content of the store was only slightly elevated Ca²⁺ release from this store was more easily initiated with low concentrations of the ER Ca²⁺ ATPase (SERCA) antagonist thapsigargin in Cln3 mutant cells. Subsequent spontaneous Ca²⁺ release events were also more common after thapsigargin addition. These findings demonstrate why CLN3 mutant cells are more sensitive to autophagy induction after thapsigargin treatment and demonstrate that CLN3 plays a role in cellular Ca²⁺ homeostasis impacting upon autophagic pathway flux(2).

In addition to this Ca²⁺ pathology we are beginning to extensively characterize dysfunction in the endolysosomal system and beyond in an attempt to identify phenotypes suitable for therapeutic screening and biomarker development. Within the endolysosomal system we have detected trafficking abnormalities and alterations in the activity of cathepsins B and L using in situ enzyme assays. Other promising phenotypes involve the use of FITC-tagged cholera toxin B subunit to study both endocytosis and levels of ganglioside GM1, processes which may be linked to dysfunctional lysosomal Ca²⁺ homeostasis. We are utilizing our experience from the study of therapy in other LSDs(3) to develop these assays for therapeutic screening and, potentially, as biomarkers for the study of any novel disease modifying therapies which may be discovered for CLN3 disease. Biochemical biomarkers and phenotypes for CLN3 are limited and needed as the search for disease modifying therapies for CLN3 disease intensifies.

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Preferred presentation

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Gene expression study in Niemann-Pick type C fibroblasts: preliminary results of a pilot study

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Niemann-Pick type C (NPC) disease is a rare lysosomal disorder due to mutations in the genes NPC1 or NPC2. Proteins NPC1 and NPC2 are essential for intracellular transport of free cholesterol. Affected individuals accumulate free cholesterol and glycosphingolipids in late endosomes and early lysosomes. So far, the pathomechanism of NPC is not fully understood. Hypotheses proposed to elucidate the pathologic events occurring in NPC cells include activated autophagy resulting in cell stress and apoptosis, imbalanced calcium homeostasis leading to block in late endosome-lysosomes fusion, oxidative stress and additionally, several cellular pathways like inflammation, intoxication with lysolipids, secondary storage of macromolecular compounds and the lack of some by-products essential for synthesis or regulation in the cell.

Aim of our study was to examine the expression levels of genes engaged in cellular metabolic pathways in cell lines obtained of NPC patients and controls.

Total RNA was isolated from 10 NPC1 patients cell lines and 9 cell lines from control persons. Biotin-labeled cRNA samples for hybridization were prepared according to Illumina's recommended sample labeling procedure. The hybridization cocktail containing the fragmented and labeled cRNA was hybridized to HumanHT-12 v4.0 Expression Bead Chip using TargetAmp™-Nano Labeling Kit for Illumina® Expression BeadChip® which enables whole-genome expression studies. Row data obtained after microarray experiments were then analyzed with the Partek Genomic Suite v6.6 software and the resulting lists of significantly and differentially expressed genes between the biological variants were analyzed in the Ingenuity Pathway Analysis.

Statistically significant alterations in expression were observed for three genes: SOD1 coding for superoxide dismutase 1 (mean fold change, MFC 2.5), CTSK coding for cathepsin K (MFC 2.5) and CTSB coding for cathepsin B (MFC 1.5). We have found that in NPC cells the up-regulated genes were related to oxidative stress, autophagy and apoptosis. These preliminary results indicate that in human beings activation of autophagy may enhance cell stress and eventually trigger the apoptotic pathway. This was already reported in NPC1 deficient mice as well as impaired proteolysis, which underlies autophagic dysfunction. Interestingly, in a murine model of acid sphingomyelinase deficiency the overexpression of cathepsins B and D were observed. Our further work will include the validation of obtained data by qRT-PCR in human NPC fibroblasts.

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The contribution of mutant gba alleles to the development of Parkinson's disease in carriers of Gaucher disease mutations

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Gaucher disease (GD) is an autosomal recessive disease resulting from mutations in the acid β -glucocerebrosidase (GCase) encoding gene, GBA, which leads to accumulation of the GCase substrate glucosylceramide. GD patients and carriers of GD mutations have a significantly higher propensity to develop Parkinson's disease (PD) in comparison to the non-GD population. This implies that mutant GBA allele is a predisposing factor for development of PD in carriers of GD mutations. We have previously shown that in cells that derived from patients of GD and carriers of GD mutations, mutant GCase molecules lead to ER stress and to activation of the ER stress response, known as the unfolded protein response (UPR). ER stress and UPR play a key function in development of PD.

We used the fruit fly *Drosophila melanogaster* to confirm that development of PD in carriers of GD mutations results from the presence of mutant GBA alleles. *Drosophila* has two GBA orthologs and each one of them has a minos insertion, which creates a deletion in the C-terminus of the protein. We generated two different *Drosophila* models for carriers of GD mutations: flies double heterozygous for the two endogenous mutant GBA orthologs and flies expressing the human N370S, L444P or 84GG mutant GCase variants. We could recapitulate UPR activation in the two different models. More so, double heterozygous flies and flies expressing the transgenic mutant human GCase variants in their dopaminergic cells developed Parkinsonian signs. They exhibited UPR, death of dopaminergic cells, shorter life span and had a decreased negative geotaxis. Flies heterozygous for only one endogenous mutant gene did not present UPR or parkinsonian signs. ER stress and Parkinsonian signs could be rescued by growing the double heterozygous flies or flies containing the N370S or the L444P mutant GCase variants in the presence of the pharmacological chaperone ambroxol, which binds and removes mutant GCase from the ER. However the flies containing the 84GG mutant variant, which does not express mature GCase, did not respond to growth in the presence of ambroxol.

Our results strongly suggest that the presence of a mutant GBA allele in dopaminergic cells leads to ER stress and to their death, and contributes to development of Parkinson's disease.

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Targeted re-sequencing in the diagnostic work-flow of neurodegenerative metabolic diseases with Mendelian inheritance – a pilot study

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We have explored the utility of targeted resequencing in the diagnostic workflow for neurodegenerative metabolic disorders in a population of patients referred for suspicion of inherited metabolic disorders in a small-scale pilot study. The patients were referred for next-gen sequencing (NGS) by four in-house metabolic specialists – paediatricians and neurologists; selected external samples were also accepted.

The initial gene panel was composed of 52 genes mutated in neuropsychiatric disease of child- and adulthood, seizures with emphasis on myoclonic epilepsy, and progressive neurological disease. While the largest group of diseases were lysosomal storage disorders, other diseases including disorders of cobalamine and amino-acid metabolism were also included. The panel was tested simultaneously with probes accumulated in other metabolic disease-oriented projects, bringing the total number of sequenced genes to more than 3,500. Sequencing was performed on the Illumina platform, the reads were mapped onto human genome (hg19) using Novoalign CS version 1.08 or later. Found variants were annotated using ANNOVAR Annotation Tool. Rare variants were evaluated using additional software tools and mutation databases.

The original aim was to shorten the way to the diagnosis by introducing NGS sequencing early in the diagnostic process, however, more often than not, we were evaluating patients in who the traditional work-flow did not lead to the diagnosis. The analysis was finished in 19 probands. The most common symptoms included psychomotor deficit, ataxia, cerebellar or brain atrophy, seizures including myoclonic epilepsy, facial dysmorphism, growth, cataracts, hypertrichosis and megaloblastic anemia. Specific suspicion (for a cobalamin metabolism disorder, Lafora disease, neuronal ceroidlipofuscinosis) was noted in five cases.

The sequencing identified pathogenic variation in malin in the patient with suspected Lafora disease, which was corroborated by finding of Lafora bodies in the skin biopsy. Two apparently pathogenic ABCD4 variants were found in a patient with megaloblastic anemia, confirming cblJ disorder. ABCD4 encodes a protein involved in lysosomal transport of cobalamin. Rare non-synonymous variants were found in COG8 (CDGIIh) in two sisters with severe psychomotor deficit, facial dysmorphism, quadraparesis, and cataracts. Other variants possibly related to proband's diseases were identified in POLG and MECP2 (non-syndromic mental retardation).

Conclusion: our limited experience suggests that targeted re-sequencing with a moderately large panel of genes may lead to diagnosis in patients with specific suspicion for a group of diseases, while it is less successful in patients in who the previous extensive biochemical and clinical evaluation did not establish the cause of the disease, where exome sequencing may be a better choice.

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Preferred presentation

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Nervous system involvement in the Fabry mouse

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Fabry Disease is caused by a deficiency of alpha-galactosidase A and is characterized by the lysosomal accumulation of globotriaosylceramide (Gb3) in most peripheral organs and tissues. Fabry patients have significant Gb3 storage in the heart, kidney and in vascular endothelial cells. As a result, patients develop cardiac disease, which often complicates progressive renal disease. The usual cause of patient death, by the age of 40-50 years, is renal failure. Unlike most other LSDs, patients do not have significant neurological involvement, because Gb3 is normally only expressed at low levels in the CNS. Although Gb3 storage has been shown to occur in some parts of the brain, most patients are generally asymptomatic with regard to the CNS. In stark contrast to the subtlety of CNS involvement, peripheral nervous system involvement is manifest from an early age in the majority of patients and indicates clinical onset of the disease. Affected hemizygotes suffer a painful neuropathy of the feet, legs and hands, predominantly, and extremal pain is the most debilitating symptom experienced by patients. Abnormal conduction times, indicative of dysfunction of large myelinated fibres and decreased thermal and vibrational sensitivity of the extremities, pointing to small fibre dysfunction are also clinical signs in Fabry patients. The mouse model of Fabry disease, generated by targeted gene disruption, has a complete lack of alpha-galactosidase A activity with significant storage of Gb3 and, to a lesser extent, other glycosphingolipids (GSLs) with terminal alpha-galactose moieties. Even though the mice exhibit significant storage of Gb3 in organs and tissues, they lack any overt pathology in the periphery. In this study, we have investigated Gb3 storage and its effects in the nervous system of the Fabry mouse. Apart from Gb3 storage in some areas of the brain and peripheral nerves, we found particularly significant storage in the cell bodies of the dorsal root ganglia (DRG). Also, different cell types within the DRG exhibit different types of storage body structures as seen by electron microscopy. On analysis of the glycan structures that are stored in the DRG, we found some unique terminally alpha-galactosylated GSLs, not seen before in any other organs and tissues. Treatment with Miglustat (NB-DNJ) is effective at reducing this storage in the DRG. To investigate further, we performed a plantar pain sensitivity test in the mice and show that they have an age-related hyposensitivity in the hindpaw and this is also responsive to treatment with Miglustat.

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In vivo studies on retaining glycosidases with fluorescent activity-based probes

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Man contains distinct glycosidases involved in metabolism of glycoconjugates. Deficiencies in some of these underlie inherited lysosomal storage disorders. Recently, fluorescent activity-based probes (ABPs) were designed at Leiden University for several retaining glycosidases. The ABPs allow very sensitive, specific visualization at high resolution of active enzymes in living cells and mice by fluorescence imaging. The developed ABPs are cell-permeable mechanism-based probes with cyclophellitol-type scaffolds that covalently link to the catalytic nucleophile amino acid residue mediating the double displacement catalysis of retaining glycosidases. The first class of ABPs consists of α - or β -glycoside configured cyclophellitol-epoxides tagged with fluorophores like BODIPY's or Cy's [1]. This class of compounds offers probes specifically recognizing glucocerebrosidase and galactocerebrosidase deficient in Gaucher and Krabbe disease, respectively. The second class of ABPs comprises α - or β -glycoside configured cyclophellitol-aziridines tagged with fluorophores [2-4]. These cyclophellitol-aziridine probes tend to label more broadly in class glycosidases. For example, while fluorescent β -glucoside configured cyclophellitol-epoxides specifically recognize glucocerebrosidase (GBA1), corresponding cyclophellitol-aziridines also label other retaining β -glucosidases like GBA2, GBA3, and LPH. Of note, the mechanism-based ABPs label their target mammalian glycosidases across species. Due to their amphiphilic nature, both types of ABPs are able to label in situ enzymes in living cells and animals. Here we describe organismal in vivo labeling of β -glucosidases with ABPs. Examples are presented for mice and zebra fish (*Danio rerio*). Illustrated is also the potential of ABPs, by virtue of being specific irreversible inhibitors, to generate pharmacological LSD models of glycosidase deficiencies in various organisms of choice.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

The V-ATPase: In vivo analysis of its contribution to (auto-)phagosome fusion and mTORC1 signalling

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A functional V-type H⁺-ATPase (V-ATPase) complex is a prerequisite for acidification of defined cellular compartments, thereby ensuring their functionality. Since the V-ATPase has also been implicated in membrane fusion events and in the signalling through the mTOR complex 1 (mTORC1), thus contributing to the regulation of autophagy, we asked in how far a lack of the V-ATPase complex interferes with these processes.

We therefore generated mice bearing a conditional knockout of the V-ATPase accessory protein 2 (ATP6AP2). Mice lacking ATP6AP2 in hepatocytes and fibroblasts show a decrease in V-ATPase assembly that leads to a massive accumulation of vesicles in hepatocytes. Electron microscopy and immunofluorescence approaches identified these as autophagosomes filled with non-degraded material, which suggests a dependence of autolysosome formation and function on the presence of ATP6AP2 and the correct assembly of the V-ATPase.

Analysing the phosphorylation of known mTORC1 regulators and downstream substrates indicated an increased mTORC1 activity in liver extracts of ATP6AP2-deficient mice. A more detailed analysis in ATP6AP2-deficient fibroblasts revealed opposing mTORC1 responses depending on the source of activation. Ongoing analyses aim to better understand the cross-talk between the V-ATPase complex and mTORC1 activity.

Mutations in Vps41, encoding a regulator of lysosomal fusion events, cause a Parkinson-like phenotype and reduction in cellular LAMP levels

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Two male children (brothers) were identified with a neurodegenerative phenotype of dystonia, ataxia and cerebellar atrophy, resembling Parkinson's disease. Exome sequencing revealed that they are both compound heterozygotes for mutations in VPS41.

VPS41 is a highly conserved protein, which was first identified in yeast as part of the HOPS (Homotypic fusion and Protein Sorting) complex, a cytoplasmic tethering complex involved in lysosomal fusion events. In previous studies we showed that depletion of VPS41 from *Drosophila* or human cells affects correct delivery of lysosomal membrane proteins (LMPs) but not lysosomal enzymes. By immuno-electron microscopy we found that VPS41 associates with late endosomes and lysosomes as well as with small transport vesicles that carry LAMP-1 and LAMP-2 but not mannose 6-phosphate receptor (MPR) from the trans-Golgi network (TGN) to late endosomes, a process that we refer to as the Vps41 pathway (Pols et al., Nat. Comm., 2013). In *Drosophila*, we showed that knockdown of Vps41 (Light) in addition to LAMPs also impairs lysosomal delivery of NPC1 (Niemann Pick C1) (Swetha et al., Traffic 2011) and our preliminary data indicate a role of the Vps41 pathway in the transport of LIMP-2, the receptor for beta-glucocerebrosidase (GBA), the causative gene for Gaucher's disease. Based on these data we propose that VPS41 regulates the transport of LAMP-1, LAMP-2, NPC-1 and LIMP-2/GBA from the Golgi to lysosomes via a pathway that is independent and complementary to MPR dependent transport of lysosomal enzymes. In addition, Vps41 as part of the HOPS complex is required for late endosome – lysosome fusion and hence delivery of endocytosed cargo to lysosomes (Pols et al., Traffic 2013).

Our preliminary studies on patient derived primary fibroblasts carrying mutations in Vps41 show that these cells have 1) a delay in delivery of endocytosed cargo to lysosomes, 2) more but smaller lysosomes, 3) no apparent defect in lysosomal degradation capacity and 4) significantly lower protein levels of LAMP-1 and LAMP-2. This phenotype is significantly different from 'classical' lysosomal storage disorders caused by mutations in lysosomal enzymes, leading to accumulations of non-digested material and swelling of lysosomes. We currently investigate whether the low LAMP levels affect LAMP-associated functions, such as lysosomal integrity, fusion capacity and chaperone mediated autophagy. Interestingly, Vps41 was recently identified as a protective factor in Parkinson disease in *C. elegans* and human model systems and mutations in NPC1, LIMP2 and GBA were all very recently recognised as risk factors for Parkinson's disease. We propose that the Vps41 pathway is important for maintaining lysosomal integrity and lipid homeostasis and that mutations in machinery or cargo of this pathway may be an important cause for Parkinson's disease.

Preferred presentation

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Intracellular lipid storage syndrome (lipidosis) following prolonged treatment of poloxamer 407 in mice

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Poloxamer 407 (P-407) is a known inhibitor of lipases (endothelial lipase, hepatic lipase and lipoprotein lipase), induced in vivo significant hyperlipidemia in mice and rats in a dose-dependent manner (Johnston, 2010). We have shown that in even relatively small dose (300 mg/kg, repeated administrations for 1 month) P-407 induced significant abnormal increase in serum total cholesterol (5-times, $p < 0,001$), LDL-cholesterol (up to 70-times) and TG (up to 30-times higher) at 24 h after administration in CBA mice with restoration at 4-7 days. The aim – to investigate lipid storage syndrome in liver cells in this model. According to a novel small-angle X-ray scattering (SAXS) method analysis for the determination of the fractional and subfractional composition of LP-C and LP-TG there was significant increase in serum LP-C, LP-TG and LP-phospholipids in P-407-treated mice. P-407 significantly increased atherogenic LDL-C fractions, as well as intermediate-density lipoprotein C (IDL-C), and LDL₁₋₃-C subfractions, and very-low-density lipoprotein-C (VLDL-C) fractions, as well as VLDL₁₋₂-C and VLDL₃₋₅-C subfractions). In general, the changes in serum lipid profile resembled the familiar hypercholesterolemia in humans but elevation was much more prominent as compared to the patients with hyperlipidemia and atherosclerosis.

In liver of mice significant increase in the total lipids concentration was noted and according to histochemistry lipids were shown in liver cells. Electron microscopic study revealed in liver macrophages massive intralysosomal accumulation of lipids.

Elevation in serum chitotriosidase (CHIT1) activity was shown which may be a sensitive indicator of macrophage involvement than cystatin C in lipidosis. Since macrophage storage syndrome of lipids can induce disturbances in lysosomal function of these cells, it may potentially play an important role in the pathogenesis of foam cell formation in liver and in atherosclerotic plaques. A drastic increase in the activity and expression of CHIT1 has been demonstrated in Gaucher's disease. It is thought that the increase in CHIT1 activity seen in Gaucher's disease is due to massive lipid accumulation in macrophages. So, repeated administrations of P-407 in mice can be used as a pharmacological model of liver lipidosis induced by P-407, general inhibitor of lipases. Since hyperlipidemia is accompanied by activation of different pools of macrophages, it would appear that the changes in serum CHIT1 activity is a potential biomarker for the development of lipid storage syndrome.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Mannose 6-phosphate-independent transport of lysosomal enzymes in liver cells

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GlcNAc-1-phosphotransferase is a hexameric enzyme complex ($\alpha_2\beta_2\gamma_2$) that catalyzes the first step in the synthesis of mannose-6-phosphate (M6P) recognition marker on lysosomal acid hydrolases required for efficient transport to lysosomes. Mutations in GNPTAB gene encoding the alpha- and beta-subunits of the GlcNAc-1-phosphotransferase associated with loss in its catalytic activity lead to mucopolysaccharidosis II (MLII, I-cell disease), characterized by missorting of multiple lysosomal hydrolases and the lysosomal accumulation of non-degraded material. Despite the lack of M6P residues, various cell types in the liver, such as hepatocytes, kupffer cells and leukocytes of MLII patients have nearly normal level of certain lysosomal enzymes. To examine the transport of lysosomal enzymes in these cell types in more detail, we used MLII mice mimicking clinical and biochemical features of the human disease.

Enzyme activity measurements of liver lysates revealed a significant increase of iduronate-2-sulfatase and alpha-fucosidase, whereas alpha-L-iduronidase and beta-glucuronidase were not impaired. Additionally, Western Blot and immunofluorescence microscopy showed M6P-independent transport of cathepsin D, cathepsin B and cathepsin Z to lysosomes.

As the most abundant cell type in liver, hepatocytes were isolated for further investigation. Western Blot, Pulse Chase and immunofluorescence analysis indicated that many lysosomal enzymes reach lysosomes M6P-independently, e.g. cathepsin D, cathepsin Z, cathepsin B, and neuraminidase I, whereas the lysosomal transport of the cholesterol-binding protein Npc2 strictly depends on M6P residues. The loss of Npc2, however, was not associated with the accumulation of non-esterified cholesterol in lysosomes of hepatocytes. The endocytosis of the M6P-containing lysosomal enzyme arylsulfatase B (ASB) used as an index protein was only slightly increased in comparison with controls, and the lysosomal proteolytic maturation and degradation of ASB was not impaired in MLII hepatocytes. These data indicate that unlike MLII fibroblasts, neither the expression nor the distribution of M6P receptors at the plasma membrane of MLII hepatocytes is significantly altered and the lysosomes are functional. Furthermore, lc3-II levels, marker of impaired autophagy, were not altered in MLII hepatocytes.

The findings suggest that MLII hepatocytes have efficient M6P-independent transport mechanisms for lysosomal enzymes to avoid disturbance of lysosome homeostasis and organ failure.

Preferred presentation
I only want to present a POSTER

Elevated plasma lyso-sphingomyelin as a biomarker for Niemann-Pick A/B disease

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Niemann-Pick disease type A and B (NPA/B) is severe neurovisceral disorder caused by mutation in acid sphingomyelinase (ASM) gene (SMPD1) leading to deficient ASM activity and accumulation of sphingomyelin (SM) in liver and spleen of patients. NPA/B are inherited as autosomal recessive traits. Clinical course of more severe NPA starts with hepatosplenomegaly followed by neurodegeneration and death at age of 2-3 years. NPB is known as a milder form with mild or no neurodegeneration but with variable clinical symptoms as hepatosplenomegaly and pulmonary involvement. Another disease category, NP type C is caused by mutations in NPC1 and/or NPC2 genes of lysosomal cholesterol transporters and only historically belongs to NP disease because of sharing moderate accumulation of SM and some glycolipids in visceral organs of patients. Deacylated sphingolipids in plasma are reported as specific hallmarks for Gaucher (lyso-glucosylceramide) or Fabry disease (lyso-Gb3Cer). Elevated lyso-SM (SPC) in DBS of NPB patients was also reported (1). Due to the fact that the SM is not increased in body fluids of NPA/B patients, we decided to test its deacylated derivative in the plasma as a possible biomarker of NPA/B. NPC plasma samples were included to our study for comparison.

For SPC extraction of acidified plasma we used the method of Murph et al (2) with 1-butanol. Analysis was performed using LC-ESI-MS/MS on Agilent 1290 UPLC system coupled to AB/MDS SCIEX API4000 tripple quadrupole tandem mass spectrometer operating in positive ion mode. SRM common product ion with 184 m/z was selected for analysis. Prior MS analysis, lipids were separated on C8-reverse phase column. Three molecular species of SPC (C18:0, C18:1 and C20:0) were evaluated and quantified using C17:1 SPC as internal standard.

We found SPC elevated in all plasma samples from type A and B patients. In most cases, there was gradual increase in the SPC level according to severity of the disease supporting the view that there is no sharp boundary between type A and type B, but a number of different intermediate variations (3). Severe infantile forms of NPA revealed at least 20-fold increase and protracted forms 6-fold increase in the plasma SPC level against controls. In patients with NPC no changes in SPC concentration were found.

In conclusion, we confirmed the plasma SPC as useful biomarker for primary diagnostics of NPA/B disease and promising marker for clinical and therapeutic studies.

This work was supported by the research project IGA MZ NT14015-3/2013 from the Ministry of Health of the Czech Republic.

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Preferred presentation

I only want to present a POSTER

Activity-based profiling of GH31 alpha-glucosidases.

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GH31 α -glucosidases are involved in several important physiological processes in the human body, ranging from food processing, ER glycoprotein processing and quality control, to lysosomal glycogen catabolism. To develop a novel research toolbox, we designed activity-based probes (ABPs) to visualize active retaining α -glucosidases. A similar design was used as earlier successfully developed for retaining β -glucosidases, comprising of an alpha-glucoside configured cyclophellitol-aziridine with attached to it fluorophores (Kallemeijn et al. *Angew. Chem.* . 2012, 51, 12529). Covalent labeling of retaining glycosidases by cyclophellitol-aziridines is thought to take place through their covalent linkage to the nucleophile amino acid residue involved in the double displacement catalysis.

Alpha-configured cyclophellitolaziridine ABPs with several attached fluorophores (BodipyGreen: JJB382; BodipyRed; JJB347; Cy5: JJB383) were synthesized and additionally those with an attached biotin (JJB384) or without any attachment. First tested was the reactivity of ABPs with recombinant human acid α -glucosidase (GAA; deficient in Pompe disease). Potent irreversible inhibition was noted at pH 4.0 (IC₅₀ values of ABPs ranging from 50 to 450 nM). Crystallographic examination of recombinant bacterial α -glucosidase provided proof for covalent binding of the ABP to the active site nucleophile residue. Next, ABP labeling of fibroblast lysates revealed that at acid pH (3.5 -6.0) potent and highly specific labeling of GAA occurs, as demonstrable by SDS-PAGE and fluorescent scanning. At pH values of 5.5 and higher, an additional protein with a MW of about 100 kDa is also labelled. To establish the identity of various labelled proteins in fibroblast lysates we employed the biotin-containing ABP JJB384, pull down and proteomics analysis. This confirmed labeling at acid pH of 70/75 kDa isoforms of GAA as well as that of ER α -glucosidase II (GANAB, 100 kDa) at more neutral pH. The specificity of labeling of active enzyme was further confirmed by noted competition by maltose and cyclophellitol-aziridine, and the lack of labeling upon prior protein denaturation. Subsequently, we examined ABP labeling of intact cultured fibroblasts: exposure to fluorescent ABPs led to labeling of both GAA and GANAB, indicating that the ABPs reach both enzymes in their respective Golgi/lysosome and ER locations. In mouse intestine, additional proteins labeled were sucrase-isomaltase and maltase-glucoamylase, two other retaining GH31 α -glucosidases. Finally, we studied Pompe materials. ABP analysis of fibroblasts of patients with infantile and adult disease revealed marked absence of mature 70/75k Da GAA with concomitant normal levels of GANAB. Thus, the ABPs may find application in laboratory diagnosis of Pompe disease. In conclusion, cell-permeable ABPs for GH31 α -glucosidases (GH31) have been developed, offering new tools for fundamental and applied investigations on these enzymes.

Production of active human lysosomal galactosidases in *Nicotiana benthamiana* plants

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Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder resulting from deficiency of alpha galactosidase A, α Gal A, hydrolyzing the α -linked galactosyl moieties from the glycosphingolipid Gb3. Enzyme replacement therapy is employed since 2001 to treat Fabry disease. Different recombinant α Gal A preparations are currently in use. A major concern is the noted induction of neutralizing antibodies against the non-self-recombinant protein in many Fabry males, generally completely lacking α Gal A. To elegantly prevent this problem application of a modified alpha galactosaminidase, Naga or α Gal B, for ERT of Fabry disease has been suggested [1,2]. α Gal B shares 46% amino acid homology with α Gal A and catalyses the hydrolysis of α GalNAc residues from glycoconjugates [12]. Additionally, it is able of cleaving terminal α -galactoses from substrates, but less effective than α Gal. Substitution of two amino acids in its active site (Serine188 to Glutamate188 and Alanine191 to Leucine191) has been reported to change its substrate specificity, increasing activity against artificial α -galactosyl substrates such as 4-methylumbelliferyl α -galactose and para-nitrophenyl- α -galactose. We here report on the successful production of active human α Gal A, Naga and modified Naga in *Nicotiana benthamiana* plant leaves via *Agrobacterium tumefaciens* transient transformation. The catalytic properties of the various recombinant enzymes are compared as well as to those of an endogenous plant α -galactosidase.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Consequences of RIP3 deficiency on the lipid profile of a mouse model of Krabbe disease

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Krabbe disease, also called globoid-cell leukodystrophy, is an autosomal recessive lysosomal disorder caused by a deficiency of galactosylceramidase. This disease affects central and peripheral nervous systems and is characterized by a loss of oligodendrocytes and myelin and infiltration of “globoid cells” (multinucleated macrophages) around blood vessels in regions of active disease. β -galactosylsphingosine, also called psychosine, presumably implicated in cell death processes, is greatly elevated. The most frequent form of Krabbe disease occurs in infancy: rapid deterioration of psychomotor functions usually leads to death by two years of age.

Necroptosis is a form of regulated necrosis stimulated by the formation of a multi-protein complex, the necrosome, of which RIPK1 (receptor-interacting serine-threonine kinase 1) and RIPK3 are important components. Recently, the generation of Ripk3 knock-out mice (Ripk3^{-/-}) has permitted facile evaluation of Ripk3 contribution to disease in diverse mouse models. Vitner et al. described attenuated neuronopathic Gaucher disease in a mouse model of Ripk3^{-/-} background (1). Furthermore, they also reported increased abundance of un-cleaved Ripk1 in twitcher (galc^{-/-}) brain, an authentic mouse model of Krabbe disease.

The aim of our study was to assess the clinical and pathological effects of the loss of one of the key mediators of necroptosis, RIPK3, in the twitcher mouse. The lipid profile of cerebellum and spleen tissue from galc^{-/-}Ripk3^{-/-}, galc^{-/-}Ripk3^{+/-}, galc^{+/-}Ripk3^{-/-} and galc^{+/-}Ripk3^{+/-} mice aged of 37-40 days was investigated by mass spectrometry (MS) - an unbiased, highly sensitive and high-throughput technique. Gas chromatography coupled to MS (GC-MS) was used to analyse total fatty acids (free fatty acids and those attached to larger lipid molecules), while intact lipids were determined by liquid chromatography-MS (LC-MS).

Multivariate analysis of both the GC-MS and LC-MS data revealed that tissues from galc^{-/-}Ripk3^{-/-} have similar lipid profiles to those from galc^{-/-}Ripk3^{+/-} mice and clearly distinct from those of galc^{+/-}Ripk3^{-/-} and galc^{+/-}Ripk3^{+/-} mice. Decreases in numerous plasmalogen phosphatidylethanolamine species, principally found in myelin, were detected in the cerebellum of galc^{-/-}Ripk3^{-/-} and galc^{-/-}Ripk3^{+/-} mice. Significantly, psychosine concentrations were elevated in the cerebellum of galc^{-/-}Ripk3^{-/-} in a similar fashion to galc^{-/-}Ripk3^{+/-}. No lipid signature differences were identified between galc^{+/-}Ripk3^{-/-} and galc^{+/-}Ripk3^{+/-} mice. These data indicate that RIP3, a key mediator of necroptosis, does not modify the pathological sphingolipid metabolism in a genetically coherent murine model of Krabbe disease.

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Preferred presentation
I only want to present a POSTER

Blood and plasma ceramide levels in mice and patients with Farber disease

Dworski S¹, Guez S², Kamani MA³, Le Faouder P⁴, Dupuy A⁴, Bertrand-Michel J⁴, Sabourdy F^{2,5}, Medin JA^{1,3,6}, Levade T^{2,5}

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Farber disease (MIM#228000; FD) is an autosomal recessive sphingolipid storage disorder due to the deficient activity of acid ceramidase, a lysosomal N-deacylase encoded by *ASAH1* that catalyzes the breakdown of ceramide. Clinically, FD is characterized by a spectrum of clinical signs ranging from the classical triad of painful and progressively deformed joints, subcutaneous nodules, and progressive hoarseness that presents in infancy, to varying phenotypes with respiratory and neurologic involvement. Many FD patients exhibit a severe phenotype fatal in early childhood. Diagnosis is based on clinical and laboratory findings by assaying the activity of acid ceramidase in leucocytes or cultured cells, and then by analysis of the *ASAH1* gene. There is currently no effective specific therapy for FD. In order to investigate the molecular pathophysiology of FD and to explore potential therapeutic strategies, a viable mouse model has been generated (Alayoubi et al, 2013). This knock-in model, which carries the *ASAH1* P361R mutation, replicates FD. Homozygous mice develop a progressive and lethal phenotype associated with macrophage pathology and dysregulated hematopoietic microenvironment (Dworski et al, 2015). The aim of this study was to analyse the ceramide composition in the plasma or whole peripheral blood (dried blood spots) of acid ceramidase-deficient mice and compare this to results obtained from FD patients. Using liquid chromatography-mass spectrometry, ceramide species of varying acyl chain length, and degree of unsaturation were quantified. Age-dependent modifications in the total contents as well as individual molecular species of circulating ceramides observed in P361R/P361R mice will be presented. Preliminary data on dried blood spots obtained from FD patients will also be presented. The potential use of these tests in terms of diagnostic/prognostic markers or follow-up for FD will be discussed. (Supported by INSERM, University of Toronto and VML)

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Preferred presentation
I only want to present a POSTER

Evaluating the Role of G Protein PEG Encapsulation of Enzyme in a MPS I Mouse Model

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Introduction

Mucopolysaccharidosis I (MPS I) is a lysosomal storage disease caused by a deficiency of α -L-iduronidase, resulting in the accumulation of dermatan sulphate and heparan sulphate within the body, which will lead to progressive multisystem and organ dysfunction. Enzyme replacement therapy (ERT) is one of the available treatments, but it cannot cross the blood brain barrier (BBB) to cure the central nervous system (CNS) diseases and some patients develop immune response that attenuates the treatment outcome. PEGylated drugs have been shown to increase circulation time and by attaching it to glutathione, which has specific and active uptake transporters expressed at the BBB, drugs may be able to cross the BBB. The effectiveness of glutathione-conjugated-PEG encapsulated Aldurazyme (lipo-ERT) in abrogating antibody responses in MPS I mice was evaluated in this study.

Methods

Two groups of MPS I mice received a weekly ERT or lipo-ERT for 10 weeks from the age of 4 weeks. A group untreated normal mice and a group of untreated MPS I mice were included as controls. Blood was drawn at different time points to determine antibody concentration using ELISA.

Results

There was no significant difference in antibody concentration in the two control groups. Both treatment groups have developed antibody, with high antibody levels in the lipo-ERT group. Median area under the curve was significantly smaller in the ERT group than the lipo-ERT group ($p = 0.05$).

Conclusions

Lipo-ERT does not reduce the antibody response in MPS I mice. Reasons for higher antibody response in lipo-ERT group still need to be determined.

Preferred presentation
I only want to present a POSTER

Characterisation of the NPC1 protein as a lipid and heavy metal transporter using bioinformatics and mutation analysis

Lloyd-Evans E

School of Biosciences, Cardiff University, UK

Niemann-Pick disease type C1 (NPC disease) is a rare neurodegenerative lysosomal storage disease characterized by ataxia, dementia hallmarks and peripheral phenotypes. NPC cells have intra-lysosomal accumulation of multiple lipids including cholesterol, sphingomyelin, glycosphingolipids and sphingosine. Lipid storage is in part a product of defective endocytosis caused by reduced intra-lysosomal Ca²⁺ levels and altered lysosomal Ca²⁺ signaling. The disease is caused by mutations in the NPC1 gene encoding a 13 transmembrane domain protein of the lysosomal membrane. Although NPC1 has homology to mediators of cholesterol homeostasis and can bind cholesterol at the N terminus the exact function of this putative lipid transport protein remains unresolved.

Using bioinformatics and mutational analysis we have further characterized the relationship between NPC1 and members of the bacterial RND permease family of which it is a member. Our analysis reveals that NPC1 has a conserved region related to transport of mycobacterial cell wall lipids that are cholesterol like. Interestingly, NPC1 is most closely related to, and shares a binding motif with, mycobacterium tuberculosis mycolic acid transporter MmpL3. In NPC1 this motif is found in the sterol sensing domain providing an ancestral connection to the transport of lipids and sterol like molecules. Mutation of this motif or addition of mycolic acids to healthy cells generates classical NPC disease phenotypes. Of greatest significance is that NPC disease phenotypes are found within granulomas of post-mortem human lung samples from patients with tuberculosis where mycolic acids are secreted by the invading mycobacterial pathogen. We have confirmed that NPC1 is the target of these lipids by identifying that one tuberculosis therapy, isoniazid, is capable of stimulating the function of NPC1 and can reduce lipid storage in some NPC1 mutant cell lines and in fibroblasts from human NPC disease carriers.

Further bioinformatics analysis has highlighted other roles for the NPC1 protein as an RND permease capable of transporting heavy metals including Zn²⁺. This we have confirmed using cells isolated from NPC variant patients (who have normal levels of cholesterol esterification) where the NPC1 mutation corresponds to a conserved residue essential for Zn²⁺ transport in the RND permease. By using these approaches we have strengthened the hypotheses that NPC1 is indeed a transporter and have discovered that this protein is involved in the pathogenesis of other human diseases and that these connections can be used to identify new therapeutic strategies for NPC disease.

References

- 1) Lloyd-Evans and Platt, Traffic, 2010

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

The mucopolipidosis type IV disease associated protein TRPML1 is a late endosomal pH regulated Ca²⁺ channel whose function is altered in Alzheimer's disease but not Niemann-Pick C

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It has been suggested that the lysosomal ion channel TRPML1, whose function is lost in mucopolipidosis type IV (MLIV), is also dysfunctional in Niemann-Pick disease type C (NPC disease)¹. This defect in TRPML1 activity in NPC disease cells is apparently caused by inhibition of TRPML1 by stored sphingomyelin. Furthermore, it has been proposed that loss of channel function can be reversed by the TRPML1 agonist ML-SA1 resulting in correction of NPC disease phenotypes¹.

Having shown that NPC disease cells have abnormally low lysosomal Ca²⁺ levels², we have investigated the mechanism leading to reduced TRPML1 activity in NPC disease. We also observe reduced TRPML1 activity in NPC disease as addition of its agonist ML-SA1 results in lower Ca²⁺ release from lysosomes. This is not the result of sphingomyelin storage, as lowering sphingomyelin storage has no effect on TRPML1 activity, but is instead a result of the reduced intra-luminal Ca²⁺ levels resulting in lower lysosomal Ca²⁺ release through lysosomal channels (TPC2). Further evidence of MLIV-like phenotypes in NPC cells, such as autofluorescence¹, are not recapitulated with no accumulation of subunit C of the mitochondrial ATPase in NPC disease cells.

In contrast to the earlier study¹ we observe no improvement in any NPC disease phenotype following incubation with ML-SA1. Lipid storage and endocytic trafficking defects remain unaffected. High concentrations of ML-SA1 are toxic to NPC disease cells, suggesting that this drug is not a viable therapeutic option.

Although we find no role for TRPML1 in NPC disease we have identified a central role in the pathogenesis of familial Alzheimer's disease (FAD)³. De-alkalinization of lysosomes via reduced vATPase function in FAD cells leads to activation of TRPML1 that empties the lysosomal Ca²⁺ store and disrupts endocytosis. These findings suggest that TRPML1 is activated at higher pH which matches our findings in MLIV disease cells where late endosomal Ca²⁺ levels are significantly elevated.

In conclusion, our work shows no role for TRPML1 in NPC disease. TRPML1 does not constitute a realistic therapeutic target for NPC disease as no MLIV disease like phenotypes are observed, instead, TRPML1 may be a more important therapeutic target for FAD.

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- 2) Lloyd-Evans E et al. Nat Med, 2008.
- 3) Lee J et al. Cell Reports, accepted for publication 2015.

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION.

Arylsulfatase K is the Lysosomal Glucuronate-2-O-Sulfatase involved in Glycosaminoglycan Degradation

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Inside the lysosome five sulfatases are known to act in the degradation of sulfated glycosaminoglycans like heparan sulfate (HS) and dermatan sulfate (DS). Recently, we characterized the novel sulfatase arylsulfatase K (ARSK) and demonstrated that ARSK is a bona fide soluble lysosomal matrix protein that co-localizes with the lysosomal marker LAMP1 and exhibits a mannose-6-phosphate containing N-glycan. ARSK showed enzymatic activity against artificial aryl-substrates at acidic pH (Wiegmann et al. 2013).

As the identity of the ARSK substrate remained unknown, we tested recombinant human ARSK activity against four synthetic disaccharides that naturally occurs during lysosomal GAG degradation. As a common hallmark, all these four disaccharides contained a terminal 2-O-sulfated uronic acid at their non-reducing ends (NRE): either the 2-O-sulfated iduronic acid units in 2-O-sulfo-iduronate-4-O-sulfo-N-actetyl-galactosamine (I2a4) and 2-O-sulfo-iduronate-6-O-sulfo-N-actetyl-galactosamine (I2a6) of DS or the 2-O-sulfated glucuronic acids derived from HS namely 2-O-sulfo-glucuronate-N-glucosamine (G2A0) and 2-O-sulfo-glucuronate-N-sulfo-glucosamine (G2S0). While both, I2a4 and I2a6, were solely desulfated at the 2-O-position of the iduronate by the well-known iduronate-2-sulfatase (IDS), G2A0 and G2S0 were exclusive substrates of ARSK demonstrating that ARSK is the long-sought lysosomal glucuronate-2-sulfatase (GDS). Due to its contribution in HS as well as DS degradation, we expect that GDS deficiency results in the accumulation of glycosaminoglycans-NREs containing 2-O-sulfated glucuronic acids. In order to confirm the physiological relevance of GDS we established a corresponding knock out mouse model that is currently under investigation regarding the manifestation of a lysosomal storage phenotype.

Preferred presentation

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Use of Ca²⁺ modulators for the treatment of Niemann-Pick type C disease

Maguire E, Adam Whittall, Jule Goike, Dr Emyr Lloyd-Evans

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Following observations by Lloyd-Evans et al., of reduced lysosomal Ca²⁺ within NPC1 cells; emerging evidence suggests Ca²⁺ modulation as an effective therapeutic strategy against NPC1 disease. In particular, the widely used natural product curcumin improves coat condition, weight loss, activity and tremor in *Npc1*^{-/-} mice¹. We have shown that this effect occurs following Ca²⁺ release from the ER rather than anti-oxidant properties, by comparing effects of curcumin with that of the curuminoid tetramethylcurcumin. Whilst both molecules possess an identical ability to reduce anti-oxidants, only curcumin treatment induces a Ca²⁺ wave within glial cells, which in turn corrects downstream NPC lipid storage.

We have also found that combining curcumin with stearic acid, or other lipid carriers that increase bioavailability of curcumin supplements, can induce cellular toxicity and reduce benefit. This explains observations by Erickson et al., 2012 that claimed a lack of efficacy of curcumin regarding the treatment of NPC2. Whilst promoting the use of Ca²⁺ modulators to treat NPC, this work highlights why care should be taken when consuming curcumin nutraceuticals.

With no clinically approved formulation of curcumin currently available, and following issues regarding its bioavailability, we began to investigate other Ca²⁺ modulators showing potential benefit regarding treatment of NPC. Despite previous claims³, the known lysosomal Ca²⁺ modulator ML-SA1, although able to initiate significant cytoplasmic Ca²⁺ elevation within glia, appears unable to correct NPC1 cholesterol storage phenotypes, and is in fact toxic to cells. We have also confirmed that other compounds, such as the adenosine-A2A receptor agonist CGS216804, whilst having no affect on cytosolic Ca²⁺ levels, is able to specifically correct the lysosomal Ca²⁺ defect within NPC1 glia, thereby reducing cholesterol storage. The widely prescribed acetylated amino acid tanganiil5 appears to work in a similar way to curcumin, correcting NPC1 phenotypes in glial cells by elevating cytoplasmic Ca²⁺. Unlike curcumin however, tanganiil appears to act via interaction with plasma membrane Ca²⁺ receptors to elevate cytosolic Ca²⁺.

Altogether, these results highlight potential benefits and pitfalls of a variety of Ca²⁺ modulators for the treatment of NPC. Current work is focused on investigating Ca²⁺ modulators in vitro and in vivo in *npc1* morphant zebrafish,. This work will hopefully provide us with a suitable drug for use in human patients.

References

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- 3) Shen, D. *et al* (2012). *Nature communications*, 3(731), 2012.
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- 5) Vibert N *et al* (2001). *European Journal of Neuroscience*, 13(4).

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

NPC1, a Lysosomal Zn²⁺ Transporting RND Permease

Maguire E, Clark E, Waller-Evans H, Lloyd-Evans E

School of Biosciences, Sir Martin Evans Building, Cardiff University, Museum Avenue, Cardiff, CF10 3AX. United Kingdom

Niemann-Pick type C (NPC) is a rare childhood disorder characterized by progressive loss of coordination, dementia and neuronal cell loss in multiple brain areas following mutations in either NPC1 (~95% of cases) or NPC2 genes.

NPC1 encodes a lysosomal transmembrane protein which when faulty results in the accumulation of multiple lipids within lysosomes. NPC1 belongs to the RND permease family¹. These proteins are known to transport several substrates including lipids and Zn²⁺. We have compared the sequences of various heavy metal transporting bacterial RND permeases to human NPC1 and found that it is related to a known Zn²⁺ transporting RND permease called ZneA. Interestingly, residues known to be important for interacting with Zn²⁺ in ZneA are conserved in NPC1. Fourteen of the disease causing mutations in NPC1 patients affect amino acids conserved in ZneA, and several of these affect amino acids that are required for Zn²⁺ binding and transport. NPC1 may therefore be the first protein identified as responsible for pumping Zn²⁺ out of lysosomes.

We have further characterized the function of NPC1 as a lysosomal Zn²⁺ transporter via inducing lysosomal exocytosis and analysis of Zn²⁺ transport across the plasma membrane. Following acidification of the extracellular medium and addition of Zn²⁺ we measured the degree of Zn²⁺ uptake into NPC1 null cells, wild-type cells and cells overexpressing NPC1. A distinct correlation was observed between Zn²⁺ transport and the levels of NPC1 protein present on the plasma membrane.

Following above observations, we examined Zn²⁺ levels using various fluorescent probes and found lysosomal storage within numerous NPC1 cells. In *Npc1*^{-/-} mice, the cerebellum is most affected with neuronal loss but some neurons in lobe 10 remain healthy. A correlation is observed between the presence of Zn²⁺ and neuronal cell death.

Finally, to illustrate the importance of Zn²⁺ to the pathogenesis of NPC disease we treated NPC1 cells with various Zn²⁺ chelators. One chelator, the natural product phytic acid, improves multiple NPC1 cellular phenotypes including lipid storage and lysosomal expansion. This suggests that Zn²⁺ storage is an early, possibly primary, pathogenic event, whilst identifying a new therapeutic avenue for NPC.

In conclusion, our work has identified that the NPC1 protein is indeed a lysosomal Zn²⁺ transporter and that lysosomal accumulation of Zn²⁺ in NPC disease cells and tissues contributes to pathogenesis of this disease.

References

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Generation of a Niemann-Pick type C1 zebrafish colony for the purposes of phenotyping and drug-screening

Maguire E, Wager K, Haslett LJ, Lloyd-Evans E

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Mutations in either NPC1 (~95% of cases) or NPC2 genes lead to the rare lysosomal storage disease (LSD) known as Niemann-Pick type C (NPC). The NPC1 gene codes for a lysosomal transmembrane protein which when faulty causes patients to present with progressive ataxia, dementia and neuronal cell loss in multiple brain areas.

A lack of therapies available to treat this disease has led to a need for new animal models, with zebrafish being a key candidate. Emerging use of zebrafish as an in vivo model for numerous neurodegenerative diseases, including several LSDs, follows observations that zebrafish genes often show a high degree of sequence identity with their human orthologs, whilst also having a brain structure very similar to our own¹.

After a short generation time (3 months), a single pair of zebrafish is able to produce up to 300 embryos weekly, are optically transparent and develop rapidly, externally to the mother. This allows for cheap maintenance, live imaging using fluorescent dyes and proteins, and rapid investigation of early developmental events, not possible with intrauterine gestation¹.

We have utilized two approaches for characterization of the role of npc1 in zebrafish, morpholinos against npc1 and pharmacological inhibitors. Zebrafish embryos treated for 5 days post fertilization (dpf) with drugs known to induce NPC1 phenotypes (U18666a, 1NMP, Ned19) show storage of NPC1 lipids including cholesterol, sphingomyelin and gangliosides when analyzed using live imaging, cryo-sectioning, thin layer chromatography and enzymatic assays. Furthermore, behavioral testing such as spontaneous coiling and touch response demonstrated movement abnormalities within npc1 fish that can be partially reversed using co-treatment with miglustat (the only currently FDA approved treatment for NPC disease). The ability to perform complex behavioral testing on zebrafish embryos further highlights their potential as a model of neurodegenerative disease. Furthermore, use of light-sheet microscopy on live zebrafish has allowed in vivo Ca²⁺ imaging, and demonstrates how the Ca²⁺ defect previously investigated within NPC1 cells and mice is also present within our disease model. This is the first confirmation that Ca²⁺ signaling abnormalities exist within any LSD animal model and are central to the pathogenic cascade.

To conclude, zebrafish represent a highly useful emerging model organism for numerous neurodegenerative diseases, including LSDs. npc1 zebrafish appear to display many of the characteristics previously observed in both humans and other animal models, validating their use as a drug-screening platform. Furthermore, their optical transparency allows for complex in vivo imaging that can confirm the presence of key pathogenic phenotypes not possible in other NPC disease animal models.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

White blood cells in Danon disease diagnostics: efficiency can be demonstrated in families affected by LAMP2 exon-copy number variations and somatic/germinal mosaicism

Majer F¹, Vlaskova H¹, Kalina T², Pelak O², Dvorakova L¹, Stolnaya L¹, Honzik T³, Palecek T⁴, Stara V⁵, Kubanek M⁶, Krebsova A⁶, Zeman J³, Sikora J¹

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Danon disease (DD) is an X-chromosome-linked disorder that manifests by mild cognitive deficit, myopathy and cardiomyopathy in male patients. The phenotype in female heterozygotes is less evident, variable and mitigated as a likely consequence of tissue-specific ratios/patterns of X-chromosome inactivation (XCI). DD is caused by mutations in the lysosomal-associated membrane protein 2 (LAMP2) gene. Majority of the known mutations abolish the protein expression due to truncation of LAMP2 open reading frame. Importantly, 10-15% of LAMP2 mutations are exon-copy number variations (eCNV) stemming from recombination events in intron 3.

LAMP2 specific DD laboratory testing relies on identification of the absence of the protein in cells/tissues and characterization of the mutation. Practically, an efficient diagnostic protocol should reflect: (i) gender of the proband/patient, (ii) tissue expression patterns of LAMP2 protein, (iii) alternative isoform splicing of LAMP2 pre-mRNA, (iv) XCI-mosaic LAMP2 expression in females heterozygous for LAMP2 mutations, (v) germinal/somatic mosaicism phenomena in de-novo mutated family members.

LAMP2 protein is ubiquitously expressed, therefore testing of its content can be easily performed in peripheral white blood cells. In fact, LAMP2 flow cytometry offers both minimal invasiveness and detection sensitivity down to 0.01% of deficient granulocytes. The latter is of critical importance in samples from suspect XCI-mosaic female patients and/or family members who are potentially germinal/somatic mosaics. As a conclusive testing technique, molecular genetic methods should universally assess full-length LAMP2 mRNA iso-forms (B, A and C) and consequently compare the abnormal findings to gDNA changes.

The outlined integrative diagnostic algorithm allows minimally invasive and efficient testing in clinically suspect individuals in whom the common qualitative PCR based methods failed to identify the mutation. Model examples of such situations will be provided in families affected by LAMP2 eCNVs.

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Preferred presentation
I only want to present a POSTER

Dysfunction of Cln3 protein impairs lysosomal and endocytic homeostasis

Schmidtke C^{1*}, Makrypidi G^{1*}, Pohl S¹, Thelen M², Storch S¹, Sylvester M², Brocke-Ahmadinejad N², Cotman S³, Jabs S⁴, Schulz A¹, Braulke T¹ *contributed equally

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CLN3 disease is a neurodegenerative lysosomal storage disorder caused by mutations in the CLN3 gene, coding for a lysosomal transmembrane protein of unknown function. The absence of functional CLN3 protein results in lysosomal dysfunction and accumulation of lysosomal storage material in neuronal and non-neuronal tissues. However, the underlying pathomechanisms remain poorly understood and it is unclear whether non-functional CLN3 protein directly impairs lysosomal homeostasis.

In the present study SILAC-based quantitative proteomics of purified lysosomal fractions of Cln3 Δ ex7/8 compared to wild-type cerebellar cells was used to analyse whether absence of functional Cln3 protein is manifested in an altered lysosomal protein composition. Based on Gene Ontology annotations, 91 proteins were assigned to the lysosomal compartment, of which 27 soluble and 7 membrane proteins were significantly altered in lysosomal fractions of Cln3 Δ ex7/8 compared to wild-type cerebellar cells. Among these, several enzymes involved in glycan and sphingolipid degradation, as well as in proteolytic processing of proteases, were predominantly decreased Cln3 Δ ex7/8 lysosomes. In addition, three cargo receptors of the endocytic pathway (mannose 6-phosphate receptor (Mpr300), low density lipoprotein related receptor 1 and 2 (Lrp1, Lrp2) were also dysregulated in Cln3 Δ ex7/8 cerebellar cells. Findings of the proteomic data were verified by Western blotting and lysosomal enzyme activity measurements. Interestingly, differences in mRNA levels of the respective genes suggested altered transcriptional regulation in the absence of functional Cln3 protein. Furthermore, internalisation assays of Mpr300- and Lrp1-specific ligands revealed that the sorting and recycling kinetics of various endocytic cargo receptors, and the subsequent delivery of their ligands is affected in cells lacking functional Cln3 protein. Ratiometric pH measurements could exclude changes in acidification to be responsible for endocytic alterations in Cln3 Δ ex7/8 cerebellar cells.

In conclusion, the data show that the malfunction of the lysosomal membrane protein Cln3 impairs the biogenesis and composition of lysosomes in a cell type-dependent manner and, moreover, affects processes of internalisation and recycling of cargo receptors and their ligands.

Preferred presentation

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Craniofacial and oral anomalies in mucopolidosis II knock-in mice

Markmann S¹, Koehne T^{2,3}, Schweizer M⁴, Muschol N³, Friedrich RE⁵, Glatzel M⁶, Amling M³, Kahl-Nieke B², Schinke T³, Bräulke T¹

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GlcNAc-1-phosphotransferase is a hexameric enzyme complex (alpha2beta2gamma2) that catalyzes the first step in the synthesis of mannose 6-phosphate (M6P) recognition marker on lysosomal acid hydrolases. Mutations in GNPTAB gene encoding the alpha- and beta-subunits of the GlcNAc-1-phosphotransferase lead to mucopolidosis II (MLII) characterized by missorting of multiple lysosomal hydrolases, their intracellular deficiency and subsequently lysosomal accumulation of non-degraded material. MLII patients show marked facial coarseness and gingival hypertrophy soon after birth accompanied with delayed tooth eruption and impacted teeth. To examine pathomechanisms of early craniofacial and dental abnormalities we analyzed MLII mice mimicking clinical and biochemical symptoms of human disease.

MLII mice have smaller skulls and thickening of the cranial vault accompanied with increased porosity of the calvaria and an increased site-specific exostosis. Elevated osteoclastogenesis leads to post-eruptive progressive alveolar bone loss after 1 months of age. Molar teeth of MLII mice show normal dentin and enamel layer, whereas the cementum layer was thicker accompanied with accumulation of storage material in cementoblasts. Histopathological examinations of the gingival tissue confirmed gingival hyperplasia in MLII mice as observed in MLII patients. Electron- and immunofluorescence microscopy, and radioactive sulphate incorporation experiments revealed the accumulation of non-degraded material e.g. fucosylated N-glycans, cholesterol and glycosaminoglycans in gingival fibroblasts, which was accompanied by the missorting of related lysosomal proteins, such as the cholesterol binding protein Npc2, alpha-fucosidase 1, cathepsin L and Z. The impairment of the gingival fibroblasts results in disorganized collagen structures in the subepithelial layer. The clinical significance of the findings has been supported by examination of four MLII patients revealing clinical, histological and ultrastructural findings that were highly similar to those in MLII mice.

The data demonstrate that the accumulation of storage material in addition to the production of dysorganized collagen matrix lead to the gingival hyperplasia in MLII. Furthermore, our study reveals alveolar bone loss and hypercementosis as so far unknown oral features of MLII. These findings suggest that GlcNAc-1-phosphotransferase represents a non-redundant regulator of teeth and jawbone homeostasis.

Preferred presentation

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Characterization of MFSD1 - a new lysosomal membrane protein - and its physiological role in the mouse

Massa López D¹, Lüllmann-Rauch R², Saftig P¹, Damme M¹

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Lysosomal exporter proteins mediate the transport of hydrolytic degradation products through the lysosomal membrane. The deficiency of some of these exporters, like sialin or cystinosin, leads to the lysosomal storage diseases Salla disease and cystinosis respectively. Of note, for a number of metabolites, like the great majority of amino acids or monosaccharides, these specific transporter proteins are unknown. Major Facilitator Superfamily Domain containing 1 (MFSD1) is a member of the MFS Superfamily, one of the two largest families of membrane transporters together with the ABC Superfamily. Despite a broad spectrum of substrates including inorganic and organic ions, nucleosides, amino acids, short peptides and lipids are known to be transported by MFS proteins, the specific substrate transported by MFSD1 remains unknown. MFS members comprise facilitators, symporters and antiporters, and the majority of them are known to contain 12 helical transmembrane domains.

Nevertheless, very little is known about MFSD1. We and others have identified MFSD1 in lysosomes by means of proteomics, [Chapel, 2013, unpublished], but a detailed characterization of Mfsd1 is lacking so far. We therefore cloned MFSD1 with an HA tag in C- and N-terminus in order to study its subcellular localization and transport, its precise topology and glycosylation. With these constructs and a newly generated MFSD1-specific antibody we confirmed its lysosomal localization on the endogenous level in cells and tissue sections of the mouse, identified a dileucine motif in the N-terminus used for the transport to lysosomes and confirmed that it has 12 transmembrane domains using selective permeabilization. Surprisingly MFSD1 is not glycosylated.

In order to decipher the physiological role of MFSD1, we generated a knock-out first conditional allele containing an IRES:lacZ trapping cassette and confirmed the absence of the protein in tissues of the KO mice. Deficiency of MFSD1 in mice triggers a liver phenotype already at 10 weeks of age, characterized by a disorganized hepatic structure, an increased number of macrophages, infiltration of neutrophils and chronic passive congestion. An increased number of megakaryocytes are found in the spleen of the affected mice.

The phenotype observed in livers of KO mice at 10 weeks of age emphasizes the importance of MFSD1 in the lysosomal homeostasis. The study of older mice is required to clarify the range of the phenotype in the liver, and further attempts are needed to unravel the substrate transported by MFSD1.

Agnès Chapel, Sylvie Kieffer-Jaquinod, Corinne Sagné, Quentin Verdon, Corinne Ivaldi, Mourad Mellal, Jaqueline Thirion, Michel Jadot, Christophe Bruley, Jérôme Garin, Bruno Gasnier, and Agnès Journet, An Extended Proteome Map of the Lysosomal Membrane Reveals Novel Potential Transporters, *Molecular & Cellular Proteomics*, 2013 12(6):1572-88

Preferred presentation
I have no preference

Lysosomal calcium signaling regulates autophagy via calcineurin and TFEB

Medina DL¹, Di Paola S¹, Peluso I¹, Armani A², De Stefani D^{2,3}, Venditti R¹, Montefusco S¹, Scotto-Rosato A¹, Prezioso C¹, Forrester A¹, Settembre C^{1,4,5}, Wang W⁶, Gao Q⁶, Xu H⁶, Sandri M^{1,2,4}, Rizzuto R^{2,3}, De Matteis MA¹, Ballabio A^{1,5,7,8}

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The view of the lysosome as the terminal end of cellular catabolic pathways has been challenged by recent studies showing a central role of this organelle in the control of cell function. Here we show that a lysosomal Ca²⁺ signaling mechanism controls the activities of the phosphatase calcineurin and of its substrate TFEB, a master transcriptional regulator of lysosomal biogenesis and autophagy. Lysosomal Ca²⁺ release via mucopolin 1 (MCOLN1) activates calcineurin, which binds and de-phosphorylates TFEB, thus promoting its nuclear translocation. Genetic and pharmacological inhibition of calcineurin suppressed TFEB activity during starvation and physical exercise, while calcineurin overexpression and constitutive activation had the opposite effect. Induction of autophagy and lysosomal biogenesis via TFEB required MCOLN1-mediated calcineurin activation, linking lysosomal calcium signaling to both calcineurin regulation and autophagy induction. Thus, the lysosome reveals itself as a hub for the signaling pathways that regulate cellular homeostasis.

Preferred presentation

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Viral-mediated gene therapy prevents disease development in ovine models of neuronal ceroid lipofuscinosis

Mitchell NL¹, Wicky HE², Schoderböck L², Barrell GK¹, Wellby MP¹, Russell KN¹, Bland R3, Hughes SM², Palmer DN¹

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Mutations in genes encoding a soluble lysosomal protein (CLN5) and a lysosomal transmembrane protein (CLN6) underlie two forms of the fatal lysosomal storage disease, neuronal ceroid lipofuscinosis (NCL; Batten disease). Although genetically distinct, the NCLs share common pathological features; progressive regionally specific neurodegeneration and accumulation of storage material in lysosomes, dominantly subunit c of ATP synthase in most forms. Currently there are no effective treatments for the NCLs. However here we demonstrate that single administration of gene therapy to pre-clinical genetically disposed sheep prevented disease development in these naturally occurring large animal models of NCL.

The effectiveness of two CNS-directed viral vector gene therapy platforms have been tested in two ovine NCL models (CLN5 and CLN6 affected sheep). Previously we showed lentiviral-mediated gene transfer to the sheep brain and in this study we subsequently validated the efficacy of adeno-associated virus serotype 9 (AAV9), showing that it mediated widespread, predominantly neurotropic, transgene expression in the sheep CNS.

Deficiencies in soluble lysosomal proteins are deemed particularly amenable to in vivo gene therapy via the normal lysosomal enzyme trafficking system and the phenomenon of cross-correction. To test this paradigm in sheep, six pre-clinical CLN5^{-/-} lambs were treated with intracerebroventricular and intracortical injections of lentiviral or AAV9 vectors encoding ovine CLN5. The treated sheep all remain alive at 18 months post-administration, exceeding the humane endpoint for untreated CLN5^{-/-} animals, and apart from delayed-onset visual deficits in the lentiviral cohort, they all demonstrate phenotypic correction, including preservation of cognitive and neurological function, and normalisation of intracranial volume compared with untreated animals. Gene therapy is now being explored in CLN5^{-/-} sheep with established disease to test the potential for amelioration in disease progression.

Defects in membrane-bound proteins are considered harder therapeutic targets. However, based on the hypothesis that the transmembrane protein is involved in the processing of a secreted protein or intercellular factor, we performed similar injections on six pre-clinical CLN6^{-/-} sheep. Only one AAV9-CLN6 injected animal maintained phenotypic correction through the 18-month follow up period whilst the other animals developed stereotypical CLN6 disease.

Neuropathological studies will be performed on trial completion, but to date we have shown encouraging results of sustained therapeutic and functional efficacy in large animal models of NCL, which hold promise for future clinical trials in human patients with CLN5 and CLN6 Batten disease.

This work was funded by Cure Kids NZ and the BDSRA

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Niemann-Pick disease type C: complex analysis of the effect of mutations in NPC1 gene on the mRNA and protein levels

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Niemann-Pick disease type C (OMIM #257220, #607625) is a rare autosomal recessive disorder caused by mutations in NPC1 (95 % of cases) or NPC2 genes that encode proteins cooperating on intracellular cholesterol trafficking in late endosomal/lysosomal compartment. Disruption of their function leads to the accumulation of unesterified cholesterol and other lipids. The principal symptoms include hepatosplenomegaly and progressive neurological deterioration. There are treatments modifying intracellular cholesterol levels in clinical use or in early phases of clinical testing. Elevation of mutant NPC1 protein expression or its stabilization using chemical or pharmacological chaperones may improve cholesterol transport capacity. This approach may prove feasible in the cases where the mutant protein has lower stability but remains partially active and possibly lead to novel treatments.

In our project we examined 29 cell lines (skin fibroblast cultures) obtained from NPC1 patients with defined genotypes. We quantified the level of transcripts using qPCR and the ratio of mutant allele expression using PCR-RFLP and NGS technology. Equilibrium amounts of immunoreactive NPC1 protein in the cultures cultivated at standard conditions and simultaneously treated with chemical chaperones, a proteasome inhibitor or lower temperature were determined by Western blotting. We also investigated the subcellular localization of the NPC1 protein compared to the localization of LAMP2 and PDI, late endosome/lysosome and ER markers, respectively. Furthermore we sequenced the NPC1 promoter region to search for rare structural variations that could influence transcription levels. The expression from the promoters carrying the found variants were assessed using luciferase reporter system.

Our results show that none of the mutations lead to a complete silencing of the transcription, the combination of two missense mutations leads to a balanced ratio of expression and combination of frameshift and missense mutation leads to the skewing of the ratio benefiting the missense mutation. Significant decline of mRNA level, unsurprisingly, usually correlates with lower amount of NPC1 protein but not vice versa. The lowest levels of NPC1 protein were detected in R1186H homozygotes (4 % compared to control) and in the combination A605Cfs*1/A1187Rfs*54 with virtually no detected protein. These data help understand the impact of individual mutations. In some of the treatment conditions we could see the elevation of NPC1 protein level. However it is not clear if this elevation has positive effect on the cholesterol transport and ameliorates the blockage. Future work will involve testing the intracellular cholesterol transport capacity.

Preferred presentation
I only want to present a POSTER

Impaired Fc-gamma and complement receptor mediated phagocytosis in Niemann-Pick disease Type C (NPC) macrophages.

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University of Oxford, Department of Pharmacology, Oxford, UK

Niemann-Pick disease Type C (NPC) is a rare, prematurely fatal lysosomal storage disorder characterized by progressive neurodegeneration, accumulation of cholesterol and multiple sphingolipids in late endosomes/lysosomes, reduced lysosomal calcium levels and a block in late endosome/lysosome fusion [1]. There is also evidence of altered innate immune responses, such as neuroinflammation, which promote disease progression [2, 3].

Phagocytosis, an innate immune effector cell activity is essential for host defence against invading pathogens and normal tissue homeostasis [4]. In order to investigate whether NPC macrophages show significantly altered phagocytic activity, we evaluated the uptake of IgG and complement opsonised sheep red blood cells and polystyrene beads of different sizes by J774 macrophages treated with U18666A to pharmacologically induce an NPC phenotype, and resident peritoneal macrophages from the *Npc1*^{-/-} mouse. We observed that in both models NPC macrophages displayed significantly reduced phagocytic uptake via both IgG and complement receptor uptake pathways, as determined by microscopic and flow cytometric analyses. These data will be considered in the context of pathological mechanisms operating in the disease and patient care and management[5].

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Preferred presentation
I have no preference

Impaired Drug Metabolism in Niemann-Pick Disease type C1 Mice

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The BALB/cNctr-Npc1m1N/J mouse model of Niemann-Pick disease type C1 (Npc1 mutant) is frequently used to evaluate new therapeutic approaches before transition to clinical trials. While testing a broad range of small molecule therapeutics in Npc1 mutant and control mice we consistently observed increased toxicity associated with drugs metabolised in the liver. By contrast drugs excreted intact via the kidney, such as miglustat, are well tolerated in the Npc1 mouse even at high doses. Consistent with these observations investigation of the P450 system revealed NPC1 dysfunction is associated with a significant reduction in the expression and activity of multiple cytochrome P450 catalysed dealkylation reactions and cytochrome C reductase in the Npc1 murine model. Four cytochrome P450 catalysed O-dealkylation reactions were measured in the Npc1 mouse at 3, 6, and 9 weeks of age, these are: MROD, EROD, PROD, and BROD (methoxy-, ethoxy, pentoxy and benzoxy-O-dealkylation reactions respectively). Npc1 mutant mice displayed significant reductions in all four enzymatic reactions at all time points tested. Interestingly, Npc1 heterozygous mice demonstrated similar enzymatic activities compared with control littermate at 3 weeks of age. However, Npc1 heterozygous mice had significant reductions in MROD, EROD, PROD and BROD by 6 weeks of age, and remained significantly reduced at 9 weeks of age. In agreement with these findings, cytochrome C reductase activity was significantly reduced at nine weeks of age in the Npc1 mutant and Npc1 heterozygous mice compared to control littermate mice respectively.

Microarray analysis was performed using cDNA isolated from livers of Npc1 mutant or control female littermate mice at 2-week intervals over the life span of the Npc1 mutant mice. Focusing on the P450 system, we identified 62 of 101 known genes of the cytochrome P450 family with reduced expression in the Npc1 mice. Forty-four of these 62 genes were down regulated at least at one time point within the life span of the Npc1 mutant mice, and 14 were significantly down regulated at all-time points. Genes belonging to subfamilies 1 to 3, which are mostly responsible for drug metabolism, were particularly affected (42 genes). We further validated a subset of these genes by qPCR. Perturbations in bile acid homeostasis are known to exist in NPC1 and could affect the function of the P450 system. As such we treated Npc1 mutant and control mice with ursodeoxycholate acid (UDCA). This treatment resulted in superphysiological increases in P450 activity and improvements in neuromuscular function of the Npc1 mutant mice. These findings have implications for drug evaluation in the Npc1 animal models and potentially the clinical management of NPC1 patients. The finding of reduced activity within the P450 system in heterozygous mice may have broader clinical implications.

Preferred presentation
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Development of an adeno-associated viral mediated gene therapy approach for Mucopolysaccharidosis IIIC

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Mucopolysaccharidoses (MPS) type IIIC is a neurodegenerative lysosomal storage disorder caused by the lack of the heparan sulphate (HS) degrading enzyme heparan sulfate acetyl-CoA:α-glucosaminide N-acetyltransferase (HGSNAT). HGSNAT deficiency affects lysosomal catabolism of HS resulting in widespread central nervous system pathology, leading to behavioural problems, including developmental delays, behavioural difficulties, sleep disturbances & dementia. The biggest challenge in developing therapies for MPS is achieving efficient delivery into the central nervous system. In MPSIIIC, the lysosomal hydrolase HGSNAT is a transmembrane protein; therefore secretion and cross-correction are unlikely. The adeno-associated viral (AAV) vector system has been used in studies for neurological correction including an ongoing clinical trial for MPSIIIA. Intracranial injection of AAV could potentially restore brain enzyme levels correcting neurological input for MPSIIIC patients. We have developed an AAV based gene therapy for MPSIIIC and have demonstrated short term efficacy in the mouse model. Intraatrial injection of AAV-HGSNAT, using 2 serotypes, Rh10 and AAV9, achieved high levels of enzyme activity throughout the brain which exceeded WT levels. The highest levels of HGSNAT enzyme activity were localised near the injection site; sections R2 and R3. AAV9 vector related HGSNAT activity was significantly higher than WT in sections R2 and R3, 355.9% and 365.8% respectively. In addition, Rh10 HGSNAT activity was significantly higher than WT levels in sections R2 and R3; 201.3% and 215.9%. AAV-HGSNAT is effective in producing enzyme activity levels exceeding normal levels in the brain, providing evidence that this is a potential treatment for the CNS involvement in MPSIIIC.

Preferred presentation

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Lysosomal cathepsins and glucocerebrosidase in Gaucher disease

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Patients suffering from the lysosomal storage disorder Gaucher disease have defects in lysosomal glucocerebrosidase (GBA1). Consequently they break down too little glucosylceramide (GlcCer) inside lysosomes of macrophages, resulting in development of characteristic pathological storage cells (Gaucher cells). GBA1 is known to be degraded inside lysosomes by cathepsins, as is indicated by the stabilizing effect of the protease inhibitors leupeptin and E64. E64-sensitive proteases include Cathepsins B, F and S. Lysosomes contain a multitude of cathepsins, a class of proteases comprising aspartyl (cathepsin D, E), serine (cathepsin A, G) and cysteine proteases (B, C, F, H, K/O, L1, L2/V, S, W, Z/X). Of note, for several cathepsins it has been found that gene expression, as well as protein and activity levels are increased in Gaucher plasma, spleen and brain tissue. More detailed knowledge on cathepsins in Gaucher disease is relevant since it might render insight in turnover of GBA1 under pathological conditions as well offer a novel avenue for therapy in which inhibition of specific cathepsins might boost lysosomal GBA1 levels by reduction of its intralysosomal turnover.

Fluorescent activity based probes (ABPs) are used to visualize lysosomal GBA1 and E64-inhibitable cathepsins. Enzymatic activity of GBA1 is measured with fluorogenic 4-MU-beta-glucoside substrate. Neutral and lysosphingolipids are analysed using HPLC and UPLC-MS based methods. CRISPR technology is utilized to specifically knock-out cysteine cathepsins.

Expression of mRNAs encoding Cathepsins D, K, S and Z/X gene expression was found to be significantly increased Gaucher versus control spleens ($p < 0.05$). The same was observed by analysis of protein levels. Inhibition of cathepsins by incubation of cells with leupeptin or E64 led to 1.5 to 3 fold increases in GBA1 protein and activity in Gaucher patient fibroblasts and lymphoblasts, as shown by increased ABP labelling and 4-MU assays. Importantly, in glycosphingolipid-accumulating Gaucher lymphoblasts a functional correction in lipid abnormality by cathepsin inhibition was observed; excessive glucosylsphingosine was lowered. The performed studies indicate that inhibition of cathepsins indeed results in functional correction of GBA1 capacity in Gaucher-derived cells. Based on inhibitor-sensitivity, likely key cathepsins involved in intralysosomal degradation of GBA1 are cathepsins B, F and S. Ongoing specific knock out of each of these cathepsins should elucidate which one initiates lysosomal breakdown of GBA1. Future development of a specific inhibitor of the relevant cathepsins is aimed for.

Preferred presentation
I only want to present a POSTER

Palmer DN

Mutations in at least 13 different genes lead to different forms of Batten disease, (the neuronal ceroid lipofuscinoses, NCLs). These inherited neurodegenerative diseases are grouped by similar symptoms and pathologies. A defining shared feature is the accumulation of fluorescent lysosome derived storage bodies in most cells. Despite ultrastructural differences, all NCLs share the common feature of storage of specific proteins. Direct sequencing of storage body proteins established specific storage of subunit c of mitochondrial F1Fo ATP synthase, first in South Hampshire sheep with a CLN6 form, and extended to CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 forms. Initially the first 40 amino acids of subunit c were determined and later the complete and normal 75 amino acid protein.

Comparing the yield of the protein sequenced with the total storage body protein loaded allowed an estimate of the proportion of storage bodies that is subunit c, as over 75% of the protein or 50% of the storage body mass. This complements the dominance of the subunit c on gel electrophoresis of total storage bodies. No evidence for a lipid peroxidation origin of the storage bodies was found nor of any intrinsic fluorophore. Reconstituted storage bodies were remarkably storage-body-like fluorescent structures when the non-fluorescent protein and lipid storage body molecules were combined.

Storage of subunit c has also been inferred from immunohistochemical staining in a large number of cases, including CLNs 2,3,5,6,7, 8 and 11. Despite the robustness of these results they are not universally accepted and unfortunately subunit c is not be detectable in many modern “universal” proteomic methods. Trypsin does not cleave subunit c and chymotryptic digestion is required to provide fragments for LC-MS verification of lysine-43 trimethylation. Subunit c has a high propensity to irreversibly aggregate, is insoluble in many solvents used in protein analyses and is insensitive to Coomassie blue staining.

A longstanding paradigm in the lysosomal storage diseases is that the storage material itself is the cause of the pathology. However storage bodies accumulate in most cells in most tissues in these NCLs, without any suggestion of tissue or organ failure or disruptions of cellular functions and the pattern of storage body accumulation is independent of the progressive regional atrophy. Neuroinflammation starts regionally, indicating that neurodegeneration and storage body accumulation are independent manifestations of the mutations. While many NCLs are linked by the accumulation of subunit c and similar patterns of symptomology and neurodegeneration the link between these two phenomena remains elusive.

Acknowledgements.

I thank Professor Sir John Walker, Ian Fearnley and associates at the MRC Mitochondrial Biology Unit, Cambridge, UK and Jaana Tynnelä and Marc Baumann, University of Helsinki collaborated on this work which was supported the US National Institutes of Health, the Batten Disease Support and Research Association and the Neurological Foundation of New Zealand.

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Desialylation of brain gangliosides by endosomal neuraminidases 3 and 4 is essential for neuronal function

Pan X¹, Velasco-Martin JP², Cairo CW³, Morales C⁴, Miyagi T⁵, Pshezhetsky AV^{1,4}

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The sialylated glycosphingolipids are the major class of glycoconjugates in neurons where they carry the majority of the sialic acid (Sia) within the central nervous system. They are essential to central myelination, to maintain the integrity of axons and myelin, and are important for the transmission of nervous impulses. By stabilizing neuronal circuits, gangliosides may have a function in memory. Neuraminidases (sialidases) that catalytically remove terminal Sia residues have been previously implicated in neuronal differentiation, neurogenesis, and axonal growth. Two of 4 mammalian neuraminidases, Neu3 and Neu4 residing on lysosomal, endosomal and plasma membranes are dominantly expressed in the brain and are primarily active against gangliosides.

To elucidate physiological functions of Neu4 and Neu3 in the brain, we generated a gene-targeted mouse strain (Neu3/Neu4 double-knockout, DKO) devoid of both enzymes. DKO mice showed normal growth, were indistinguishable from WT animals and could be bred to produce knockout litters, but pathological examination of the brain showed that approximately 5% of the deep cortical neurons, adjacent to the hippocampus presented a mild accumulation of lysosomal bodies. Similarly, the epithelial cells lining the choroid plexus presented an accumulation of small vacuoles at the base of the cells also indicative of lysosomal storage. The sialidase activity measured with gangliosides as substrates in the brain tissues of Neu4/Neu3 DKO mice was reduced to <10%, indicating that Neu3 and Neu4 are the neuraminidases mainly responsible for desialylation of brain gangliosides. This was consistent with progressive accumulation of GM3 ganglioside in the brain tissues of DKO mice as compared to WT and single Neu3 or Neu4 KO mice detected at 10 months by thin-layer chromatography. Immunohistochemical analysis of brain sections showed that gangliosides mainly accumulated in hippocampal neurones. To determine if these changes in neuronal morphology and ganglioside composition affected CNS function, we performed behaviour studies, and detected that DKO mice have abnormal alteration pattern in Y-maze and Novel object recognition tests suggesting deficits in spatial and short-term memory, respectively. The difference with WT and single Neu4 or Neu3 KO mice was observed as a trend at 6 month, but became statistically significant at 10 months.

Together our data for the first time provide evidence for that Neu3 and Neu4 have partially overlapping roles in regulation of brain gangliosides essential for CNS function.

Preferred presentation

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Mucopolysaccharidosis IIIA Storage Substrate Drives an Innate Immune Neuro-inflammatory Response

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Mucopolysaccharidosis type IIIA (MPSIIIA) is a paediatric lysosomal storage disease characterised by mutations in the sulfo-glucosamine sulfo-hydrolase (SGSH) gene, resulting in reduced lysosomal SGSH enzyme activity. Subsequently, an accumulation of highly sulphated, partially degraded heparan sulphate oligosaccharides occurs in lysosomes and the extracellular matrix with disease progression, alongside secondary accumulation of GM gangliosides, cholesterol and amyloid beta. MPSIIIA patients develop behavioural disturbances and progressively worsening cognitive deficits, ultimately leading to dementia and premature death, a possible consequence of neuro-inflammation. Brains from MPSIIIA mice demonstrate markedly increased astrogliosis and microgliosis, particularly in the cortex (Wilkinson et al., 2012). This, coupled with up-regulation of IL-1 α , IL-1 β and TNF α , suggests the development of a pro-inflammatory environment in MPSIIIA. However, the molecular mechanisms responsible for neuro-inflammation in MPSIIIA remain unclear. This project aims to understand how HS and secondary storage substrates affect the CNS and neuro-inflammatory pathways.

Here we show that glycosaminoglycans (GAGs) isolated from MPSIIIA mice induce pro-inflammatory TNF α , IL-6, IL-1 α and IL-1 β responses when applied to a primary mixed glial culture, where normalised levels of WT GAGs did not elicit a response. The data also shows that qualitative alterations, rather than amount, in MPSIIIA GAGs are responsible for inflammatory responses. MPSIIIA GAGs act as an inflammatory priming stimulus via toll-like receptor 4 (TLR4) as inhibition of the intracellular domain of TLR4 completely abrogated the inflammatory response ($p \leq 0.001$). MPSIIIA GAGs did not initiate IL-1 dependent signalling alone in this in vitro model; indeed recombinant IL-1 receptor antagonist (IL-1Ra) did not reduce the inflammatory response. Secondary stimulation with NLRP3 inflammasome activators such as ATP or secondary storage substrates accumulated in MPSIIIA (monohydrate cholesterol crystals or amyloid beta oligomers) was required to induce the release of intracellular IL-1 β associated with MPSIIIA GAG priming ($p \leq 0.001$).

In vitro data suggests that MPSIIIA neuro-inflammation may be dependent on IL-1, and driven by primary and secondary storage substrates. We are currently performing in vivo studies to confirm whether the MPSIIIA neuro-inflammatory response acts through IL-1 dependent mechanisms, and whether modulation of the innate immune response will slow disease progression.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Measurement of lysosphingolipids and their isoforms by LC-MS/MS in plasma, urine and amniotic fluid: application to screening of sphingolipidoses

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Lysosphingolipids (LSL) are emerging markers for the diagnosis and follow-up of sphingolipidoses. Their measurement has been settled-up in plasma by liquid chromatography/tandem mass spectrometry (SSIEM, Lyon 2015, oral communication). Existence of isoforms of LSL in urine/plasma has been demonstrated by others.

On these bases, we have undertaken the estimation of many possible isoforms of LSL in plasma, urine and amniotic fluid (AF) of controls and sphingolipidoses affected patients.

- Analysing plasma LSL on small plasma samples in a single run is efficient for the diagnosis of several sphingolipidoses, showing:
 - elevated lysoglobotriaosylceramide in Fabry disease,
 - moderate increase of lysohexosylceramide (LHexCer) in Krabbe disease (KD, n=4) and high increase in Gaucher disease (GD, n=2) and saposin C deficiency (n=1),
 - increase of lysosphingomyelin (LSM) in Niemann-Pick (NP) type A/B (n=8).
 - abnormal presence of lysoGM1 and lysoGM2 gangliosides in GM1 gangliosidosis (n=2) and Sandhoff d
- Further analysis of isoforms patterns allows:
 - to distinguish in vitro KD (one isomer of LHexCer increased) from GD (seven isoforms highly increased).
 - simultaneous screening of NPA/B (14 isoforms highly increased) and NPC (n=11, plasma LSM slightly increased, but seven isoforms highly increased).

Plasma isoforms were also increased in Fabry disease and GM1/2 gangliosidoses. Only a plasma marker for metachromatic leukodystrophy is lacking.

- In urine, LSL and their isoforms could also be increased in the corresponding disorders. Patterns were often different from plasma. Thus, the metabolism of LSL and their isoforms is to be investigated.
- LHexCer and seven isoforms were dramatically increased in AF of fetuses affected with severe GD, allowing the early and rapid screening of antenatal presentations of GD.

Thus, LSL measurement provides a first-line simultaneous screening of sphingolipidoses and could replace classical screening methods (such as enzymatic measurements, filipin staining). Numerous isoforms observed could represent new biomarkers for these disorders for the diagnosis and follow-up of sphingolipidoses, opening the way to “lysosphingolipidomics”.

Preferred presentation
I have no preference

Differential Inflammasome Dysregulation in Lysosomal Storage Diseases

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Inappropriate activation of the innate immune system, in the form of non-limiting inflammation, is a shared characteristic of lysosomal storage diseases (LSDs). Neuroinflammation is prominent in all disorders that have pathology within the CNS and is recognized to actively promote disease progression. Anti-inflammatory drugs have been shown to provide benefit in a number of LSD models. However, we still have a relatively poor understanding of the mechanisms that induce and the precise nature of pro-inflammatory responses in specific LSDs.

The prototypic pro-inflammatory cytokine IL-1 β is produced via the activities of cytosolic protein complexes known as inflammasomes. Generation of bioactive cytokine requires two signals; a priming signal that induces transcription and an activation signal that triggers inflammasome complex assembly and enzymatic cleavage of the pro-cytokine to yield mature IL-1 β .

We have begun an investigation into the contribution of IL-1 β in LSDs. We have found evidence of differential dysregulation of NLRP3 inflammasome activity in individual sphingolipid storage disorders. We will present data revealing that whilst in a murine model of Sandhoff disease there is inappropriate activation of NLRP3 inflammasome and enhanced generation of IL-1 β , in murine Niemann Pick type C disease inflammasome activity is impaired and production of the cytokine significantly reduced. These results illustrate that the inflammatory profile of LSDs differs between disorders and is very likely to influence anti-inflammatory strategies that may be applicable in the clinical setting.

Preferred presentation

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Lysosomal system as a hub for copper sensing and homeostasis in health and disease

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Copper (Cu) is an essential nutrient, which is required as a cofactor for a number of enzymes operating in respiration, neurotransmission, connective tissue biogenesis and pigmentation. On the other hand, red-ox properties of this metal constitute a serious threat for the cell viability. Cu toxicity fully manifests in Wilson disease, a genetic liver disorder caused by metal overload due to mutations in Cu transporter ATP7B. Therefore, organisms evolved finely tuned mechanisms for Cu sensing, intake, storage and consumption.

In vertebrates ATP7B traffics towards canalicular/apical area of hepatocytes to remove excess Cu into the bile. Despite the importance of this process, the trafficking mechanisms of ATP7B remain poorly understood. Here we show that in response to elevated copper ATP7B moves from the Golgi to lysosomes and imports metal into their lumen for transient storage. ATP7B also enables lysosomes to undergo apical exocytosis and therefore to release stored copper into bile. This exocytic process is triggered by the copper-dependent interaction between ATP7B and p62 subunit of dynactin that allows lysosomes to move along the microtubule tracks towards the canalicular pole of hepatocytes. Transcriptional activation of lysosomal exocytosis significantly increases ATP7B delivery to the canalicular membrane and copper clearance from the hepatocytes. Suppression of ATP7B leads to lysosome stress that manifests in abnormal morphology and function of the organelles. Moreover, lack of ATP7B in the lysosome compartments triggers signaling mechanism, which involves TFEB-mediated transcriptional activation of autophagy and contributes to Wilson disease pathogenesis.

Our findings indicate that lysosomes serve as an important intermediate in Cu sensing and homeostasis and can be targeted for novel therapeutic approaches to combat Wilson disease.

Preferred presentation

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Synaptic Defects in Mucopolysaccharidosis IIIC Mouse

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Severe progressive neurological pediatric disease, Mucopolysaccharidosis III type C (MPS IIIC) is caused by mutations in the HGSNAT gene leading to deficiency of acetyl-CoA: α -glucosaminide N-acetyltransferase involved in the lysosomal catabolism of heparan sulfate. The cause of the major clinical manifestation of the disease, rapid mental decline, is still not well understood. The mouse model of MPS III C generated in our lab by germline inactivation of the Hgsnat gene in C57Bl/6 mice mimicked the human disease progression (Martins et al., 2015). At 6-8 months mice showed hyperactivity, and reduced anxiety followed by cognitive memory decline at 10 months. Neuronal loss became significant at 10 months and further progressed with age but was not a dominant feature at the early stage of the disease.

To test the hypothesis that cognitive dysfunction and other manifestations occurring in the MPS IIIC mice prior to the age when significant neuronal loss is observed are associated with a decrease in neurotransmission we analyzed synaptogenesis, synaptic spine density and morphology in the CA1 region of the hippocampus using MPS IIIC mouse model that expresses GFP in hippocampal neurons. We found that in the MPS IIIC mice the spine density and maturity were decreased already at the age of 20 days and further declined with age. Similar decline in spine density was observed in the hippocampal neurons of a mouse model of another lysosomal disorder, Tay-Sachs disease indicating that synaptic defects are caused by lysosomal storage in general. The formation and trafficking of synaptic vesicles in the neurons was further analyzed by immunohistochemistry using antibodies against several pre- and post-synaptic markers involved in docking and fusion of synaptic vesicles. The levels of both synapsin and synaptophysin, were decreased in cultured hippocampal neurons from MPS IIIC mice compared to those from wild type animals, as well as the levels of the pre-synaptic proteins VAMP2 and synaptophysin in brain sections. Besides, neuroligin-1 crucial for synaptic spine formation was accumulated in perinuclear structures in the soma (likely lysosomes or late endosomes) of the MPS IIIC neurons instead of being localized in synaptic spines as it occurred in wild type cells. Mutations causing missfolding and misslocalization of neuroligin and its binding partner β -neurexin lead to defects in synaptic transmission causing cognitive diseases such as autism (Sudhof 2008). We speculate that abnormal localization of neuroligin-1 in MPS IIIC cells may also affect its interaction with β -neurexin compromising synaptic activity and brain function. Together our results document synaptic defects on both hippocampal and cortical neurons presumably leading to the cognitive deficiencies in the MPS IIIC patients.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Osmotic behavior of lysosomes as an index of autophagy in cellular lipid overloading and experimental treatment of early senescence

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Autophagy is an universal cellular response on insufficiency of living conditions (Ravikumar et al., 2010). Regulation of autophagy includes its self-inhibition because hyper-stimulated segregation of own cell constituents is malicious. The regulation consists of up- and down-phases (Yu et al., 2010), and stimulation of autophagy in the up-phase can be therapeutically positive, but can be fatal in the down-phase. It is especially important for the cells of nerve system (unable to regeneration by proliferation) for which autophagy is the main mechanism of resistance against neurodegeneration and survival. This concerns directly LSD since neurodegeneration is the main pathology in many lysosomal diseases. Thus, testing of autophagy, its up- and down-phases is important for understanding of autophagy therapeutic abilities. But this self-regulation is not absolute and further stimulation of autophagy can lead to cell death (Yu et al., 2010). We applied here an osmotic test for autophagy estimation since it reflects physiological state of lysosomal compartment connected with active enzymatic degradation of segregated material and was recommended not so long ago for autophagy study (Klionsky, 2008). Briefly, the test includes osmotic damage of lysosomes by hypotonic medium (2x dilution) and estimation of release of β -galactosidase (lysosomal lumen enzyme marker). Existence of two phases of autophagy regulation was studied in an experiment with starvation. One-day starvation of mice led to reliable increase of osmotic susceptibility of liver lysosomes (from 21.2 to 30.3 %), while two-days starvation results in decrease of the index (23.5 %) nearly to control level. Seemingly it reflects self-inhibition of autophagy at the late term of starvation and should be confirmed by electron microscopy (in process). In the next experiment with poloxamer P-407 inducing hyperlipidemia and liver cells lipid storage syndrome development we found permeabilization of lysosomal membranes and increase of osmotic susceptibility of lysosomes. Possibly, accumulation of lipid droplets in cytoplasm can stimulate autophagy including segregation of these lipid droplets. The model reflects abundant situations of liver lipidosis. Finally we studied a possibility to influence on autophagy in premature senescence since cell senescence and LSD are connected with decrease of autophagy. The activation of autophagy in these situations seems important for future therapeutic interventions. Brain front cortex and hippocampus of rats Wistar (as control) and OXYS (premature senescence) under application of neuroactive ceftriaxone have been studied. Osmotic susceptibility of the samples was nearly the same for Wistar and OXYS rats. But application of ceftriaxone led to elevation of osmotic test values for OXYS rats without any effect on Wistar rats (substantially for front cortex). The index failed to discriminate autophagy activity between Wistar and OXYS rats but was useful in revealing autophagy stimulating activity of ceftriaxone for OXYS rats that can be exploited in further basic research.

Preferred presentation

I only want to present a POSTER

Enzyme-loaded nanoparticles: a potential therapy for the neurological compartment in Mucopolysaccharidosis type II

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Mucopolysaccharidosis type II (MPSII) is a lysosomal storage disorder due to the deficit of the enzyme iduronate 2-sulfatase (IDS), leading to the accumulation of the glycosaminoglycans heparan- and dermatan-sulfate in most organ-systems, including the brain. Main treatment, represented by the weekly infusion of the functional enzyme, cannot help the neurological involvement, affecting about 2/3 of the patients, due to the inability of the enzyme to cross the Blood-Brain Barrier (BBB).

Here, polymeric nanoparticles (NPs), modified with a glycopeptide of 7aa (g7), already tested for the BBB transport of low molecular weight molecules, were tested as a possible vehicle for the transport to the CNS of high molecular weight molecules, as the recombinant therapeutic enzymes, after systemic administration in mice. Following a preliminary assessment using albumin as model drug, an in vivo feasibility study was conducted in the MPSII mouse model testing the g7-NPs loaded with the recombinant IDS enzyme. This study confirmed that the g7-NPs are able to carry the enzyme over the BBB, opening the way to the efficacy studies, performed both in vitro and in vivo.

In the in vitro study, fibroblasts from MPSII patients were treated for 7 days with NPs loaded with IDS (g7-NPs-IDS). Although the activity induced by NPs resulted much lower than that obtained after administration of the purified enzyme, we measured enzyme levels similar to those detected in healthy cells and sufficient to reduce GAG content to non-pathological levels.

To evaluate the efficiency of transfer and the enzyme activity induced by g7-NPs-IDS in a more complex physiological system, an in vivo pilot evaluation as well as a medium-term study were conducted in the MPSII mouse model. In the former, mice treated with a single dose and sacrificed after 7 or 14 days, showed no induced IDS activity or reduction of GAG deposits in both liver and brain. Instead, in the medium-term analysis, we conducted in the mouse model weekly administrations of g7-NPs-IDS for a total of 6 weeks. Biochemical, histological, immunohistochemical and immunofluorescence evaluations in the liver and in the brain of treated mice suggested that a period of 6 weeks of treatment, although still insufficient to allow massive release of the encapsulated enzyme from NPs, is sufficient to produce a significant reduction of the GAG deposits and general improvement in the brain and in the liver of treated mice.

Given the positive results obtained, we are re-formulating NPs with the aim to maintain the observed good transport efficiency to the CNS, but allowing a reduction of the timing of drug release.

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Preferred presentation
I have no preference

Intravenous AAV9-mediated gene transfer prevents pathology in neonatal Sandhoff mice

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Sandhoff disease or GM2 gangliosidosis variant 0 is a genetic disorder due to mutations in the HEXB gene. It is characterized by a double Hex A ($\alpha\beta$) and B ($\beta\beta$) deficiency, responsible for a GM2 accumulation, mainly in the central nervous system (CNS). Clinically, the disease begins in the first months of life and culminates in death around 3 years of age. Up to date, no specific treatment is available for Sandhoff disease. A murine model obtained by invalidation of the Hexb gene is a useful tool for the development of therapeutic approaches.

An AAV9-Hexb vector placed under the control of the phosphoglycerate kinase (PGK) promoter was constructed and intravenously administered in neonatal Hexb^{-/-} mice. Two different doses were tested, but the most efficient was 3.5 x 10¹³ vg/kg. Treated animals have a survival similar to normal mice (>700 days) by comparison with naïve Sandhoff mice only living approximately 120 days. At the end of the study, injected mice were phenotypically non distinguishable from normal mice. They were active, they have no loss of weight, no tremor or ataxia. Behavioral tests were performed regularly using rotarod, activimeter and inverted screen test and AAV9-treated mice showed results comparable to controls.

Hexosaminidases activities were tested at 2 and 4 months in treated mice by comparison with naïve Sandhoff and normal mice. Assays were performed by using the artificial substrates permitting to analyze either the Hex A and B or the specific Hex A activities. Total hexosaminidases were not restored to normal after intravenous administration of the AAV9-Hexb, but they reached a potentially therapeutic level. Hexosaminidase A was around 15 % of normal into the brain and 40% in the liver. These activities were still significant at 24 months. Some other lysosomal enzymes were found increased in Hexb-deficient mice. Their activities were significantly lowered in AAV9-treated mice at 2, 4 and 24 months.

Ganglioside accumulation was evaluated in brain tissue after lipid extraction, ganglioside purification and high-performance thin-layer chromatography. Brain GM2 storage found in untreated Sandhoff mice was absent in normal as well as in AAV9-treated Sandhoff mice at 2 and 4 months post-injection. The cerebellum was analyzed separately showing a huge but incomplete GM2 reduction.

Different immunohistochemical methods were used to study brain lesions. Lamp-2 staining showed a significant storage in Sandhoff mice, but not in AAV9-treated and control mice. Astrocytosis, microgliosis and neuron loss were found in naïve Sandhoff mice, but they were absent in AAV9-treated mice. These results suggest a protective effect of the therapeutic vector administered intravenously in affected mice during the neonatal period.

Preferred presentation
I have no preference

Lysosomal dysfunction disrupts presynaptic maintenance in neurodegenerative diseases through a α -synuclein- and CSP α -dependent pathway

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Lysosomes are degradation organelles, which are mainly involved in cellular protein homeostasis and whose dysfunction is associated to several neurodegenerative conditions. Here we identified a disease-relevant link between lysosomal dysfunction and presynaptic proteostasis maintenance. In mouse models of lysosomal storage disorders (LSDs), a group of inherited neurodegenerative diseases characterized by a global lysosomal impairment, and in cultured neurons treated with lysosomal inhibitors, lysosomal pathology develops progressively with alterations in presynaptic structure and function. In these models, impaired lysosomal activity leads to a concomitant presynaptic loss of α -synuclein and cysteine string protein- α (CSP α), which, in turns, severely deregulates the proteostasis of soluble NSF attachment receptor (SNARE) at nerve terminals. Overexpression of CSP α in the brain of LSD mice efficiently re-established SNARE-complex levels, thereby ameliorating presynaptic function and attenuating neurodegenerative signs. Our data unveil a new role of lysosomes in maintaining presynaptic integrity and demonstrate that disruption of α -synuclein- and CSP α -dependent SNARE proteostasis at nerve terminals is a key pathway by which lysosomal dysfunction drives neurodegenerative processes.

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Preferred presentation
I have no preference

The role of the lysosomal intramembrane protease SPPL2a in the regulation of immune cell signaling

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Signal Peptide Peptidase-like (SPPL) 2a is an intramembrane cleaving protease which is localized in the membrane of lysosomes and late endosomes. It mediates the intramembrane cleavage of various type II transmembrane proteins, thereby liberating a cytoplasmic intracellular domain (ICD) from the substrate protein, which can migrate to the nucleus and influence transcriptional regulation [1]. We recently identified CD74, the invariant chain of the MHCII complex, as a major substrate of SPPL2a. In SPPL2a-deficient mice, an N-terminal fragment (NTF) of CD74 accumulates in B cells and provokes a maturation arrest of these cells [2]. We found that signal transmission via the B cell antigen receptor (BCR), which is essential for developing B cells, is significantly impaired in SPPL2a^{-/-} B cells. Particularly, tonic activation of the PI3K/Akt pathway is disturbed by the accumulating CD74 NTF accounting for reduced phosphorylation of the transcription factor forkhead box subgroup O 1 (FoxO1) and enhanced expression of pro-apoptotic genes [3].

Beyond the described indirect effects on signalling pathways by CD74, we report that certain members of the C-type lectin receptor family of pattern-recognition receptors are proteolyzed by SPPL2a and/or its plasma-membrane resident homologue SPPL2b. Dectin-1 is a receptor for fungal β -glucans on dendritic cells and macrophages. After ligand-binding, this protein is rapidly internalized and degraded in the lysosome [4]. We found that internalization of Dectin-1 induced by its endocytosed ligand Zymosan led to the generation of a Dectin-1 NTF in transfected HEK cells, which is further processed by co-expressed SPPL2a/b proteases. Most importantly, inhibition of endogenous SPPL2 proteases significantly stabilized the Dectin-1 NTF and thereby enhanced receptor mediated signaling in various cell types and conditions. Our data surprisingly reveal a so far unknown role for SPPL2 proteolytic activity in terminating Dectin-1 signaling highlighting these proteases as novel modulators of pattern recognition receptor signaling and anti-fungal immunity.

In conclusion, our studies reveal that intramembrane proteolysis in the lysosomal membrane can significantly impact on signaling in immune cells in a positive or negative way, based on the responsible substrate protein and the underlying mechanism.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Enzyme Replacement Therapy of Metachromatic Leukodystrophy: Nanoparticles fail to deliver Arylsulfatase A across the Blood-Brain Barrier

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Metachromatic leukodystrophy is a lysosomal storage disease (LSD) caused by deficiency of arylsulfatase A (ASA). Intralysosomal accumulation of 3-O-sulfogalactosylceramide (sulfatide), a component of myelin and substrate of ASA, leads to progressive demyelination, resulting in severe neurological symptoms and premature death. For MLD and other LSDs with prevailing CNS disease, enzyme replacement therapy is forestalled by the blood-brain barrier, preventing intravenously injected ASA from reaching the brain parenchyma. In order to increase ASA-transfer across this barrier, we aim at using surface modified ('surfactant coated'), polymeric, biodegradable nanoparticles as ASA carriers. Upon intravenous injection, surfactant coating has been shown to recruit apolipoproteins, facilitating receptor-mediated transcytosis of the nanoparticles across brain endothelial cells (Kreuter et al., 2001; Petri et al., 2007).

In principal, there are three strategies to use nanoparticles as drug carriers: (1) drug incorporation during their production (polymerization), (2) drug adsorption or (3) covalent / high affinity binding on their surface. Incorporation of ASA into poly(butylcyanoacrylate) (PBCA) or poly(lactic acid) nanoparticles led to a complete loss of ASA activity. In contrast, ASA could be efficiently adsorbed to the nanoparticle surface without significant loss of activity. However, upon incubation in serum surface-bound ASA completely dissociated within seconds, prohibiting use of this nanoparticle formulation in vivo. Therefore we developed strategies in which ASA was bound covalently or with high affinity to nanoparticles to prevent desorption in serum. Using amine-reactive crosslinking chemistry, we were able to covalently couple sufficient amounts of ASA to poly(human serum albumin) (HSA) nanoparticles. For non-covalent, high affinity coupling, neutravidin was covalently attached to PLA nanoparticles, to which biotin labelled rhASA was bound. Both formulations were tested for their pharmacokinetics upon tail-vein injections in mice. Contrary to our expectations, neither ASA-nanoparticle formulation led to elevated ASA levels in the brain in comparison to injections of free ASA. Instead, the biodistribution was only altered among peripheral organs.

Preferred presentation
I only want to present a POSTER

Stable isotope labelling with amino acids in cell culture (SILAC): A proteomics approach to compare the cell-surface interactomes of alpha-mannosidase and arylsulfatase A

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Enzyme replacement therapy (ERT) has been proven safe and effective in several lysosomal storage diseases (LSDs). However, for LSDs with prevailing central nervous system (CNS) manifestation the blood brain barrier (BBB) prevents efficient transfer of recombinant lysosomal enzymes from the blood circulation to the brain parenchyma and thereby limits benefit of ERT. This holds true, for example, for metachromatic leukodystrophy, a LSD caused by functional deficiency of arylsulfatase A (ASA). Alpha-mannosidosis represents an exception in this regard as ERT was effective in improving CNS storage and functions in (pre)clinical ERT studies (Blanz et al., 2008; Borgwardt et al., 2013), confirming data from bone marrow transplantation experiments (Walkley et al., 1994). The observed CNS effects could be due to higher transcytosis rates of alpha-mannosidase across the BBB in comparison to other lysosomal enzymes.

To address this hypothesis, we compared the cell-surface interactome of recombinant human lysosomal alpha-mannosidase (rhLAMAN) and rhASA using stable isotope labelling with amino acids in cell culture (SILAC). This approach involves following steps: (i) metabolic labelling of cultured cells with ^{14}N , ^{12}C - (light) or ^{15}N , ^{13}C - (heavy) arginin and lysin, respectively, (ii) enrichment of plasma membranes by concanavalin A affinity chromatography, (iii) solubilization of plasma membrane proteins, (iv) affinity chromatography of light and heavy labelled protein fractions using immobilized rhLAMAN and rhASA, respectively, (v) elution of bound proteins and identification by mass spectrometry.

In membrane protein fractions prepared from human non-endothelial cells (HeLa), the cation-independent mannose-6 phosphate receptor (MPR300) was found to bind to rhASA with much higher efficacy than to LAMAN (heavy/light ratio of 10.6). In contrast, clusterin (apolipoprotein J), a soluble protein interacting with members of the LDL receptor family, was among the proteins binding predominantly to rhLAMAN (heavy/light ratio of 0.23). In membrane protein fractions of the murine brain endothelial cell line bEND.3 neither MPR300 nor clusterin bound to immobilized rhASA or LAMAN, in agreement with a low expression of the two proteins at the BBB. Instead the platelet endothelial cell adhesion molecule 1 (PECAM-1) was identified as a protein binding to immobilized rhLAMAN, but not to rhASA.

To investigate consequences of potentially different affinities to MPR300 on the endocytic rate, the uptake of rhLAMAN and rhASA was analyzed in vitro. In agreement with the SILAC data, endocytosis of rhLAMAN was significantly lower compared to ASA in HeLa cells. Notably, endocytosis of both enzymes could be inhibited by mannose-6 phosphate.

Our results suggest that rhLAMAN and rhASA interact with different cell surface proteins. The low affinity and cellular uptake of rhLAMAN by the MPR300 may favor transfer across the BBB by unspecific and/or specific processes mediated, e.g., by binding to cell surface proteins of brain capillary endothelial cells such as PECAM-1.

Preferred presentation

II only want to present a POSTER

Bone Infiltration of Gaucher Disease Evaluated by Bone Marrow Burden Score: evolution after one year of treatment

Paskulin L^{1,2}, Camargo M¹, Bertholdo D², Vanz AP², Vairo F¹, Vedolin L³, Schwartz JD^{1,2,4}

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Introduction: Bone involvement of Gaucher disease (GD) results from Gaucher cells infiltration in the bone marrow, causing bone necrosis and osteoporosis. The extent of it can be evaluated by Magnetic Resonance Imaging (MRI), via Bone Marrow Burden score. Objectives: to report the evolution of bone disease in patients with GD after one year of treatment. Methods: Serial MRI were performed, the first one at Time Zero (BMB1) and the second after one year of Time Zero (BMB2) at the Gaucher Disease Reference Center of Rio Grande do Sul – Brasil. The MRI images were quantified according to the BMB score: values between 0-2 indicate absence of bone infiltration; 3-7, mild infiltration; 8-12, moderate infiltration; and 13-16, severe bone infiltration. To perform the analysis of the data, patients were classified into two groups: Group 1 included patients that were already being treated before Time Zero; Group 2 included patients that had started the treatment at the same time as Time Zero. Results: 27 patients were studied, 3 patients had Type III GD, the rest of them had Type I GD. Twenty two patients were included in Group 1 and five patients in Group 2. Group 1: one patient was being treated with Substrate Reduction Therapy (SRT) with Miglustat, six patients were being treated with Enzyme Replacement Therapy (ERT) with alfa-Taliglucerase, and 15 with Imiglucerase. The median time receiving treatment before the beginning of the study was 9.25 years. BMB median score was 8. There was no difference between BMB 1 and 2. The median dose of ERT by Time Zero was 25UI/kg/inf and remained stable after one year. Group 2: two patients started SRT with Miglustat, one started ERT with alfa-Velaglicurase and two with Imiglucerase. BMB1 median score was 13. The median dose of ERT by Time Zero was 15UI/kg/inf. BMB2 median score was 10. The median dose of ERT was stable after one year of treatment. Spearman test showed no statistical correlation between BMB score and ERT dose (correlation coefficient = -0.251 and p = 0.092) nor between BMB score and time receiving treatment (correlation coefficient = -0.088 and p = 0.549). Discussion: there was no reduction of BMB score among the patients of Group 1. There was a reduction of BMB score among patients of Group 2; among these, 3 had no reduction or increase in their BMB score (2 received treatment with Miglustat and 1 with Imiglucerase 15UI/kg/inf); 2 patients had their BMB score significantly reduced (1 received ERT with alfa-Velaglicurase 60UI/kg/inf and 1 with Imiglucerase 15 UI/kg/inf). Conclusion: only with a larger longitudinal study it will be possible to correlate clinical data with bone involvement on GD patients. The bone infiltration appears to respond more abruptly at the beginning of the treatment, and it appears to not modify after clinical stabilization of GD.

Preferred presentation

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Identification of Recombinant Alleles in GBA1 Gene in Patients with Neuronopathic and Non-neuronopathic Gaucher Disease

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Introduction: Gaucher disease (GD), an autosomal recessive genetic disorder, is caused by deficient activity of the glucocerebrosidase enzyme due to pathogenic mutations in the GBA1 gene. This gene comprises 11 exons and has a pseudogene (GBAP) with 96% of sequence homology (1). Recombination (Rec) events in the GBA1 seem to be facilitated by an increased degree of homology and proximity to the GBAP, leading to gene conversion, fusion or duplication. The L444P mutation is the second most common pathogenic variant in GBA1, and it may occur alone or in cis with other mutations. Many protocols of genetic analysis for GD patients include only the investigation of the most frequent mutations, which prevents the differentiation between L444P alleles and those resulting from recombination events (2). Among the most prevalent complex alleles is the RecNcil, which includes 3 distinct mutations located at exon 10 (L444P, A456P and V460V) of GBA1. The aim of this study was to identify the presence of recombinant alleles in GBA1 in patients with DG known to be L444P carriers in at least one of their alleles. **Methods:** Twenty-two unrelated GD patients (type I = 17; type II = 3; type III = 2) followed by the GD Reference Center in Rio Grande do Sul, Brazil, were included in our sample group and had their exons 10 and 11 of GBA1 sequenced. L444P mutation was previously identified in 26 out of 44 alleles (59%). **Results:** Recombinant alleles were present in 14 out of 26 (53.8%) L444P alleles, corresponding to an allele frequency of 31.8% (n=14/44). Twelve (85.7 %) out of 14 Rec alleles were RecNcil. Among those RecNcil alleles, 7 also had the g.7668G>A variant and 6 out of 7 also had the g.7678T>C alteration, both located in the 3'UTR. The allele frequency of Rec variants was 64.7%, 100% and 0% in GD patients type I, II and III, respectively. **Conclusion:** The present study highlights the importance of sequencing the whole GBA1 gene, considering that at least half of L444P alleles are complex ones. The identification of recombinant alleles may contribute to a better understanding of genotype-phenotype correlation in GD.

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Mucopolidose II and III Alfa/Beta no Brasil: atualização sobre a análise genética do GNPTAB

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Introdução: Mucopolidose II e III são distúrbios recessivos autossômicos caracterizados pela alteração do transporte e localização subcelular de enzimas lisossômicas devido à deficiência da glicosiltransferase 1-fosforil, que adiciona um marcador chave responsável pelo reconhecimento do resíduo de manose 6-fosfato que permite a entrada das hidrolases lisossômicas no lisossomo. A glicosiltransferase 1-fosforil é codificada pelos genes GNPTAB (ML II ou III alfa/beta) e GNTPG (ML III gama). **Objetivo:** Caracterizar o GNPTAB em 9 pacientes brasileiros com ML II/III. Para um paciente adicional (paciente 10), sem amostra de DNA disponível, o DNA da mãe foi analisado. **Metodologia:** De 9 pacientes, três (e a mãe do paciente 10) tiveram todos os 21 exons do GNPTAB sequenciados. Para os demais pacientes (n=6), o GNPTAB foi sequenciado de acordo com o protocolo desenvolvido pelo nosso grupo (passo 1= exon 19; passo 2= exons 13 e 10; passo 3= exons 3, 12, 13.2, 14 e 20; passo 4= 1, 2, 4-9, 11, 15-18 e 21). **Resultados (Tabela 1):** Cinco pacientes foram clinicamente diagnosticados como tendo ML II, e cinco, como tendo ML III. Em 9/9 pacientes, ambas as mutações patogênicas foram identificadas. O diagnóstico foi realizado no primeiro passo para 1/6 paciente, no segundo passo para 2/6 pacientes, no terceiro passo para 2/6 pacientes e no quarto passo para 1/6 paciente. A mãe do paciente 10 foi encontrada como portadora da mutação c.3503_3504delTC. A mutação patogênica mais frequente foi a c.3503_3504delTC, encontrada em 8/19 alelos, em heterozigose composta (n=4 pacientes) ou homozigose (n=2 pacientes). Duas mutações novas foram encontradas: c.1154C>T (p.S385L) e c.1924_1927delAATT (p.N642LfsX10). A mutação p.S385L não foi encontrada em 100 controles saudáveis e no Projeto Genoma 1000, e o SIFT e Polyphen-2 preveem que se trata de uma mutação patogênica. Como esperado, pacientes homocigotos para mutações nonsense/deslocamento de leitura apresentaram o fenótipo mais severo (ML II), e pacientes com mutações pontuais, ML III. **Conclusão:** ML II/III alfa/beta parece ser o tipo mais comum de ML no Brasil. Nossos dados estendem o conhecimento sobre o espectro de mutações no GNPTAB e podem contribuir para a compreensão das correlações genótipo-fenótipo em pacientes com ML II/III alfa/beta.

Preferred presentation
I have no preference

Neonatal bone marrow transplantation prevents bone pathology in a mouse model of mucopolysaccharidosis type I

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Bone marrow transplantation (BMT) performed in neonatal age could offer a new therapeutic opportunity for inborn errors of metabolism, since the supply of the missing protein at birth could prevent tissue damage. We tested this hypothesis in Mucopolysaccharidosis type I (MPS IH, Hurler syndrome), a lysosomal storage disease caused by deficiency of α -L-iduronidase (IDUA) enzyme, characterized by progressive skeletal abnormalities and other clinical manifestations (1). Current BMT approaches increase the lifespan of MPS IH patients, but musculoskeletal manifestations are only minimally responsive to the treatment (2). We hypothesized that the transplantation of normal bone marrow (BM) into newborn MPS I mice, soon after birth, could be effective in preventing skeletal dysplasia.

The transplantation of murine wild-type BM into busulfan-conditioned MPS I neonates led to high long-term engraftment levels (3). A >50% engraftment resulted in an increase of IDUA activity in organs and in the clearance of glycosaminoglycans (GAGs) from blood and tissues. At 37 weeks of age, the reconstitution of a normal hematopoiesis in MPS I mice guaranteed a consistent amelioration of bone pathology. Radiographs showed that the width of the long bones (femur, tibia, humerus and radius-ulna) of neonatally-treated MPS I mice significantly decreased, compared to the ones of untreated MPS I mice. Notably, the magnitude of improvement correlated with the extent of engraftment. Furthermore, micro-CT attested that several 3D-parameters of the femurs, such as trabecular number and separation, cortical thickness, and bone mineral volume, significantly differed between treated and untreated affected mice. Histologically, in untreated MPS I mice, osteocytes were increased in number and contained GAG vacuoles, while in transplanted mice these features were corrected. Overall, our evaluations confirm that BMT performed at a very early stage of life is able to prevent the symptoms of MPS I.

This is a proof-of-concept study showing that neonatal BMT could be an effective therapeutic approach for Hurler syndrome and suggesting that an early treatment may further affect the clinical outcome of these patients.

We are now developing a strategy for the neonatal transplantation of murine cord blood in the MPS I model. We aim to investigate the characteristics and the potential of this alternative hematopoietic source, which has shown a number of clinical advantages in the treatment of Hurler patients (4).

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Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Acid ceramidase deficiency (Farber disease) – behavioral, biochemical and cellular CNS pathologies in Asah1P361R/P361R mice

Sikora J^{1,2}, Dworski S³, Jones EE⁴, Kamani MA⁵, Micsenyi MC², Le Faouder P⁶, Bertrand-Michel J⁶, Dupuy A⁶, Dunn CK⁵, Xuan I³, Casas J⁷, Fabrias G⁷, Hampson DR³, Levade T^{6,8}, Drake R⁴, Medin JA^{3,5}, Walkley SU²

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Farber disease (FD, acid ceramidase (ACD) deficiency) is a lysosomal storage disorder caused by ASA1 mutations. ACD converts ceramide(s) (Cer) to sphingosine (Sph) and fatty acid(s). The majority of FD patients present with progressive visceral and neurological disease that classically associates with early-onset subcutaneous nodules, arthritis and laryngeal hoarseness. Asah1P361R/P361R mice recapitulate visceral and bone marrow pathology comparable to that in FD patients. To explore the neurological deficits, we investigated biochemical, and cellular abnormalities in the central nervous system (CNS) of the mutant mice and also tested their behavioral phenotype(s).

Cer, hydroxy-Cer (Cer-OH), dihydro-Cer, Sph, dihexosyl-Cer, and GM3 were all elevated by LC-MS. These sphingolipid classes constituted a significantly greater percent of total brain lipids, with a concomitant reduction in the proportion of monohexosyl-Cer and sphingomyelin. The most accumulated was Cer-OH, increasing >100-fold with individual chain length isoforms elevated > 40-fold. We also demonstrated specific neuroanatomical compartmentalization of the abnormal lipid profiles by high-resolution MS imaging. Cerebral and cerebellar cellular abnormalities in the mutant mice combined lysosomal storage changes in a multitude of CNS cell types and extensive secondary/reactive changes. FD storage profiles were identified in neurons and oligodendroglia, but also in vascular endothelial cells as well as in choroid plexus. However, storage laden microglia/macrophages represented the dominant neuropathological feature in the mutant mice. At end-stage (10 weeks of age), the abnormal macrophages formed coalescing and mostly perivascular granuloma-like accumulations located preferentially to white matter, periventricular zones and meninges. Neurodegeneration could be identified in specific cerebral neuronal sub-populations. Interestingly, cerebellar pathology lacked signs of patterned loss of Purkinje cells. While not differing in weight, a substantial fraction of mutant mice developed hydrocephaly. In behavioral tests, mutant mice presented with reduced voluntary locomotor and exploration activities, increased thigmotaxis, abnormal spectra of basic behavioral activities, impaired muscle grip strength and also defects in motor coordination and motor learning.

Summarized, our studies in Asah1P361R/P361R mice provide the first detailed insights into CNS pathology and pathogenic cascades stemming from ACD deficiency and could prove valuable for design of experimental FD therapies.

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

Hyperammonemia induces hepatic autophagy and enhancement of autophagic flux improves clearance of ammonia in vivo

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Systemic ammonia levels are elevated in patients with acute and chronic liver diseases, or urea cycle disorders. High levels of blood ammonia result in life-threatening brain damage and current treatments for hyperammonemia are limited. Ammonia has been recently found in cell cultures to stimulate autophagy. Therefore, we investigated whether autophagy is also induced in vivo and whether modulation of autophagy plays a role in the severity of hyperammonemia. We confirmed that ammonia induced autophagy in HeLa cells and we found that it stimulates nuclear translocation of TFEB, a master regulator of autophagy and lysosomal biogenesis. Next, we showed that acute intraperitoneal (i.p.) challenge with ammonia or chronic dietary administration of ammonia in wild-type C57BL/6 mice induced hepatic autophagy, as shown by increased LC3-II and Atg5, and reduced p62 levels in livers. Suppression of autophagy by colchicine or vinblastine significantly impaired the clearance of ammonia following i.p. ammonia challenge. Furthermore, TFEB liver-specific knockout mice showed a defect of ammonia handling compared to control mice. Taken together, these results suggest that ammonia-induced autophagy in liver is required for efficient clearance of ammonia. Moreover, enhancement of autophagy by liver-directed gene transfer of TFEB mediated by systemic intravenous injection of a helper-dependent adenoviral vector improved the ammonia clearance after ammonia challenge. Importantly, ammonia handling was also ameliorated by rapamycin and Tat-beclin 1, an autophagy-inducing peptide. The enhancement of liver autophagy was associated with increased ureagenesis. In addition, in wild-type C57BL/6 mice, rapamycin reduced the hyperammonemia induced by diet enriched with ammonium acetate. In summary, these data indicate that autophagy is an important mechanism for ammonia detoxification and could explain the well known effect on hepatic proteolysis induced by ammonia. Enhancement of autophagy by gene transfer or small molecules can alleviate both acute and chronic hyperammonemia and these treatments have potential for therapy of hepatic encephalopathy or urea cycle disorders.

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

New roles of OCRL in lysosomal function and autophagy

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OCRL is a PI(4,5)P₂ 5-phosphatase that is defective in Lowe syndrome, a rare X-linked disorder characterized by renal Fanconi syndrome, congenital cataracts, and psychomotor impairment. OCRL has been shown to control endocytic uptake and recycling, endosome-to-Golgi transport, early phagocytic steps, cytokinesis, and cilium formation. However, many gaps remain in our understanding of the mechanisms linking OCRL mutations to the manifestations of Lowe syndrome. In an attempt to fill these gaps we adopted an unbiased approach to uncover how cells suffer from/react to the loss of OCRL by analyzing the changes in gene expression caused by the depletion of OCRL. Unexpectedly, one of the gene classes most significantly up-regulated by OCRL depletion was that of lysosomal genes. Prompted by these results we investigated the role of OCRL in lysosomal function. We found that, while at steady state, as reported, OCRL associates with clathrin-coated vesicles, early endosomes, and the Golgi complex, it translocates to lysosomes under conditions demanding high degradation efficiency (such as autophagy). Interestingly, the lysosomal translocation of OCRL is insensitive to the activity state of mTORC1 (a lysosomal based signaling complex) but is controlled by Toll Like Receptor 9 signaling from autolysosomes and is mediated by AP2 and clathrin, two binding partners of OCRL. By confining in time and space the lysosomal PI45P₂ pool, OCRL safeguards the activity of mucopolipin1 (MCOLN1), the lysosomal calcium channel required for autophagosome-lysosome fusion. The elevation of lysosomal PI(4,5)P₂ caused by OCRL depletion/mutation impairs MCOLN1 activity and lysosomal calcium release, hampering the autophagic flux through lysosomes and leading to accumulation of autophagosomes. Importantly, boosting the activity of MCOLN1 with selective agonists rescues lysosomal function in kidney cells from Lowe syndrome patients, thus identifying MCOLN1 as a possible drug target. In conclusion, we have uncovered a lysosomal “cargo-load response” that is mediated by TLR9 signaling and that enhances the degradative flux through lysosomes.

Preferred presentation

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Origin of lysosomal α -mannosidase in cerebrospinal fluid

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In the last few years lysosomal enzymes have been investigated for their potential role as biomarkers in the diagnosis of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Dementia with Lewy bodies (DLB). Increased levels of the lysosomal proteases, cathepsin D and cathepsin B, have been detected in cerebrospinal fluid (CSF) and postmortem brain of AD patients. Higher activities of the lysosomal β -galactosidase and cathepsin E were found in CSF of PD patients than in controls. Other studies have reported the reduction of β -glucocerebrosidase activity in CSF of patients affected by PD and DLB and the increase of β -hexosaminidase activity in CSF of PD patients.

The differences in the enzyme activities observed in CSF of AD, DLB, and PD patients compared to controls could be the result of the altered lysosomal system that occurs in the brain. Nevertheless, the situation in which the CSF mirrors the pathological conditions that take place in the brain is only a hypothesis, since the origin of the lysosomal enzymes present in CSF is still unknown.

Lysosomal enzymes in the CSF could result directly from the secretory processes that occur in the brain tissue during the transfer of these enzymes from the Golgi apparatus to the lysosomes. However, it is also possible that they are secreted from other tissues and reach the central nervous system through the plasma flow across the blood-brain barrier (BBB). Among lysosomal enzymes α -mannosidases occur in multiple forms in human tissues and body fluids with multiple functions in glycoprotein metabolism. Lysosomal α -mannosidase activity with acidic pH optimum is ubiquitous in human tissues where it occurs as two major forms, A and B, that can be separated by ion-exchange chromatography on DEAE-cellulose. In addition in human plasma and serum the predominant form is an α -mannosidase with pH optimum of 5.5, called intermediate form. In this work the α -mannosidase activity in human frontal gyrus, cerebrospinal fluid and plasma has been analysed by DEAE-cellulose chromatography to investigate the origin of the α -mannosidase activity in CSF. The profile of α -mannosidase isoenzymes obtained in CSF was similar to that in the frontal gyrus but different from that in human plasma. In particular the two characteristic peaks of lysosomal α -mannosidase, A and B, were present in both the frontal gyrus and CSF. Furthermore the results show that the intermediate form of α -mannosidase is not present in the CSF. These results suggest that the intermediate form of α -mannosidase in plasma does not cross the BBB and that the α -mannosidase activity present in the CSF is of lysosomal type and of brain origin. Thus the α -mannosidase activity in CSF might mirror the brain pathological changes linked to neurodegenerative disorders such as PD.

Preferred presentation
I only want to present a POSTER

Changes in the lysosomal proteome after MG132 treatment

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The autophagy-lysosome and the ubiquitin-proteasome system are the two main degradative pathways of the cell. It has already been reported that an inhibition of the proteasomal degradation by proteasome inhibitors like MG132 leads to an increase in autophagy. Substrates that are normally degraded by the proteasome are directed to lysosomal degradation by this mechanism. Interestingly, it has recently been demonstrated in *Arapidopsis* that inactive proteasomes are degraded by autophagy in a process termed proteaphagy, mediated by the ubiquitin receptor RPN10. How this process is regulated in mammals is yet unknown.

To investigate the influence of proteasomal inhibition by MG132, we have used stable isotope labelled HEK cells (Arginine Arg/Arg(¹³C₆¹⁵N₄) and Lysin Lys/Lys(¹³C₆¹⁵N₂)) for a comparative analysis of lysosomes isolated by dextran-coated magnetic particles by mass spectrometry. While the statistical analysis of the data showed no proteins significantly downregulated by at least 25% in the lysosomal fractions after MG132 treatment, we found 179 significantly upregulated proteins in the lysosomal fraction by 25% or more. Among these proteins, we found all subunits of the 20 S Proteasome, as well as several subunits of the 19 S Proteasome including mammalian RPN10. These results could be verified by western blotting of lysosomal fractions, whereby in input fractions, protein levels remained unchanged. In addition, GABARAPL2, a member of the mammalian Atg8 family, was 4-fold upregulated. In immunofluorescence, GABARAPL2 showed a clear formation of a punctate staining colocalizing almost completely with LAMP2 after MG132 treatment of HEK and also NIH3T3 cells.

These results support the hypothesis that also in mammalian cells, proteasomes inactivated by MG132 treatment are degraded by the autophagic-lysosomal pathway. Further experiments to identify possible cargo receptor for mammalian proteaphagy are under way.

Preferred presentation

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GALNS mRNA aberrant transcripts due to atypical splice site and deep intronic mutations in Morquio A patients

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Mucopolysaccharidosis IVA (Morquio A syndrome) is an autosomal recessive lysosomal storage disease, caused by the deficiency of the enzyme N-Acetylgalactosamine-6 sulfatase (GALNS), involved in the catabolism of keratan sulfate and chondroitin-6-sulfate. Diagnosis is commonly based on urinary excretion of chondroitin/keratan sulfate, decreased GALNS enzyme activity in vitro followed by molecular analysis of the GALNS gene.

Accurate and efficient diagnosis of Morquio A can be challenging. Here we report the RNA sequence analysis of 5 patients with clinical and biochemical diagnosis of Morquio A, in whom the standard sequencing procedures failed to identify the second disease-causing mutation. Large deletions/duplications have been excluded by QF-PCR and CNV molecular techniques in all these patients. m-RNA analysis were necessary to complete the molecular characterization.

RT-PCR and Real Time PCR analysis were performed in biological samples from such patients identifying aberrant mRNA transcripts due to noncanonical acceptor/donor splice site mutations and by one deep intronic variant located in intron 8 of GALNS gene.

We want to emphasize the importance of analysing mRNAs to provide comprehensive GALNS diagnostic tests, to facilitate genotype/ phenotype correlations and to eventually better target the available therapy.

Towards a Metabolomic Assay of Monosialoganglioside Turnover in Normal and GM1 Gangliosidosis Fibroblasts with High Resolution Q-Exactive Orbitrap LC/MS

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Lysosomal beta-galactosidase (b-Gal) cleaves beta-linked galactose residues from GM1 ganglioside, oligosaccharides and keratan sulfate. Over 170 mutations in its structural gene (GLB1) have been identified in patients. They result in infantile GM1 gangliosidosis with progressive neurodegeneration and skeletal dysplasia as well as in attenuated phenotypes, such as juvenile GM1 gangliosidosis (juvGM1). Searching for therapeutic options, some mutant GLB1 enzymes showed increased activity against synthetic b-Gal substrate if certain N-acyl derivatives of desoxygalactonojirimycin (DGJ) were present. Since currently no appropriate assay is available to monitor intracellular turnover of fatty acid-specific isoforms of gangliosides we aim to develop a metabolomic LC-MS assay to meet this demand.

Fibroblasts from a healthy subject (WT) and a patient with juvGM1 were cultured in the presence of bovine GM1 gangliosides containing equal amounts of C:18 and C:20 fatty acids in their N-acyl moiety. Cellular gangliosides were isolated by solid phase extraction and subjected to HPLC-MS in a Thermo Scientific Q-Exactive Orbitrap System using the SIM multiplexing MS mode. Concentrations of N-acyl isoforms of GM1, GM2 and GM3 were estimated using deuterated standards.

To evaluate ganglioside uptake, cells were cultured in the presence of 1-5µg of GM1 for 4d. A dose-dependent increase of GM1 C18:0 was detected in WT and juvGM1 fibroblasts which was elevated ≤6-fold in latter cells. Next, a chase experiment was conducted growing cells in the presence of 1µg GM1 for 4d followed by a change to normal medium. Cells were harvested at different time-points to measure GM1 C18:0. Five hours after GM1 removal, juvGM1 contained 6-fold higher levels of GM1 C18:0 than WT cells. Both cell types returned to pre-load levels within 10h. To monitor the turnover of ganglioside species, cells were exposed to 1µg GM1 for 30-240min. No significant concentration difference of GM1 C18:0 could be observed in either cell type. GM3 C18:0 remained close to detection limits while GM2 C18:0, the specific product of lysosomal β-Gal-activity, was slightly elevated in juvGM1 cells. Analysis of all detectable isoforms after a 1h GM1 load revealed a prominent change in isoform patterns, probably due to transesterification of exogenous GM1 C18:0. While GM1 C16:0 was elevated in both cell types with a more pronounced difference in juvGM1 fibroblasts, GM2 C16:0 and C24:0 were significantly elevated (≤60-fold) in juvGM1 despite the deficiency of lysosomal b-Gal. GM3 C16:0, C22:0 and C24:0, in contrast, were higher in WT than juvGM1 cells. No significant changes of isoform patterns were found after 4d of GM1 exposure.

Taken together, our method successfully separates N-acyl isoforms of GM1, GM2 and GM3 and provides a possibility to determine their handling in cells. Further experiments are currently conducted to elucidate cellular pathways involved in the turnover of exogenously added gangliosides in WT and juvGM1 fibroblasts.

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Oral Eliglustat for Gaucher Disease Type 1: Dosing by CYP2D6 Metabolizer Status

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Gaucher disease type 1 (GD1) is a multi-systemic lysosomal disorder resulting from a deficiency of acid β -glucosidase. Eliglustat, an oral substrate reduction therapy recently approved for adults with GD1, is metabolized mainly by CYP2D6, a polymorphic cytochrome P450 isozyme. Clinical studies used a dose titration scheme (range: 50 mg to 150 mg, twice daily [BID]) to ensure that plasma eliglustat steady-state pre-dose concentrations (C_{trough}) were above a predefined target concentration based on in vitro inhibition data. However, this method has limitations in real-world clinical practice settings. An alternative CYP2D6 phenotype dosing strategy was proposed based on modeling approaches. For CYP2D6 intermediate metabolizers (IMs)/extensive metabolizers (EMs), the proposed dosing regimen of 100 mg BID was based on a pharmacokinetic/pharmacodynamic (PK/PD) modeling approach using population-PK model predicted plasma exposure parameters of eliglustat, and observed efficacy from eliglustat clinical studies. This analysis was sponsored by Genzyme, a Sanofi company. In a Phase 3, randomized, controlled study (ENCORE, NCT00943111, n=106 in eliglustat arm) of GD1 patients switching from enzyme replacement therapy (ERT) to eliglustat, 60 (63%) of 95 CYP2D6 IM/EM patients were dosed at either 50 or 150 mg BID (maximum dose allowed) based on C_{trough} levels during the 12-month primary analysis period. Modeling predicted that the change from baseline in spleen volume (primary endpoint) was similar whether non-treatment naïve patients were dosed by C_{trough} levels (-5.96%, observed) or if all CYP2D6 IM/EM patients had received a 100-mg BID dose (-6.63%) instead, and that this dosing regimen would not result in any additional failures to meet the primary endpoint. For all CYP2D6 IM/EM patients, projected eliglustat exposure at the 100-mg BID dose was within the range observed in the clinical studies (i.e., would not be expected to affect safety). In another Phase 3, randomized, placebo-controlled study (ENGAGE, NCT00891202, n=20 in eliglustat arm) of treatment-naïve GD1 patients, the majority (17/19, 89.5%) of the CYP2D6 IM/EM patients were successfully dosed at 100 mg BID (maximum dose allowed). This is important from a clinical perspective because untreated Gaucher patients have a higher disease burden than patients stable on ERT. For CYP2D6 poor metabolizers (PMs), the proposed dosing regimen of 100 mg once daily (QD) was based on physiologically based PK simulations. Ten virtual trials with 36 PM subjects/trial receiving eliglustat for 8 days were simulated using the SimCyp Population Based Simulator. The mean predicted exposure was shown to be within the range demonstrated to be safe and effective in the clinical development program. Overall, the proposed simplified dosing regimen for eliglustat based on CYP2D6-genotype-predicted phenotype (100 mg BID for CYP2D6 IM/EM patients and 100 mg QD for CYP2D6 PM patients) would achieve exposure, efficacy, and safety results similar to a dosing regimen based on plasma eliglustat concentrations.

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I have no preference

Pharmacogenetics Can Identify Fabry Patients Eligible for Treatment with Migalastat, and Indicate that the Majority of Migalastat-amenable Patients Have Mutations Associated with Classic Disease

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Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations in the GLA gene that lead to deficient α -galactosidase A (α -Gal A) activity. Over 800 GLA mutations have been reported; ~60% are missense. The mutant enzymes often have reduced stability and inefficient lysosomal trafficking, leading to substrate accumulation (globotriaosylceramide, GL-3; globotriaosylsphingosine, lyso-Gb3), cellular dysfunction, and disease. The orally available pharmacological chaperone migalastat (1-deoxygalactonojirimycin, AT1001) selectively binds to some endogenous mutant forms of α -Gal A, increasing their physical stability, lysosomal trafficking, and cellular activity. We assessed whether pharmacogenetic information could be used to identify FD patients that may benefit from migalastat therapy. To this end, an in vitro “GLP-HEK” assay was developed to measure increased cellular mutant α -Gal A activity after incubation with 10 μ M migalastat (~plasma C_{max} following administration of 150 mg to humans). To date, 531 FD-associated mutant forms have been expressed in HEK-293 cells. Mutants showing a ≥ 1.2 -fold relative increase and a $\geq 3\%$ wild-type absolute increase were considered ‘amenable’ to migalastat therapy. The predictive value of the assay was assessed based on pharmacodynamic responses to migalastat in Phase 2/3 studies. A high degree of consistency was observed between the GLP-HEK and white blood cell α -Gal A responses from male patients (sensitivity=1.0; specificity=0.875; positive-predictive-value=0.9460; negative-predictive-value=1.0). Comparisons of GLP-HEK assay results to clinical responses on kidney GL-3 and plasma lyso-Gb3 showed high concordance (sensitivity 0.9286-1.0; specificity 0.6875-1.0; PPV 0.8387-1.0; NPV 0.8462-1.0). In amenable patients switched from ERT to migalastat, plasma lyso-Gb3 remained low for ≥ 18 months; levels increased in patients with non-amenable mutations. Hence, the GLP-HEK assay has high predictive value in identifying migalastat-eligible FD patients. Lastly, a database of ~800 FD-associated GLA mutations was compiled that included all known mutation types (i.e., missense, small insertions/deletions, splice-site mutations, etc.). The analysis also included categorization of mutations as classic or non-classic (i.e., late-onset, variant) based on published literature. Importantly, a majority (~68%) of all amenable mutations, including those represented in migalastat clinical studies, are associated with classic FD

Preferred presentation

If selected by the organisers, I would prefer to give an ORAL PRESENTATION

The lysosomal targeting of acid alpha-glucosidase

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Lysosomal acid alpha-glucosidase (GAA) degrades glycogen to glucose and its deficiency in lysosomes results in the lysosomal storage disorder Pompe disease. Newly synthesized GAA is phosphorylated on its high mannose-type N-linked glycans, a process that ensures targeting from the trans-Golgi network to the endo-lysosomal system by the mannose 6-phosphate receptors. However, we and others have found that the lysosomal targeting of GAA is not strictly dependent on the mannose 6-phosphate targeting system, as fibroblasts from mucopolipidosis type II patients that are deficient in this pathway display normal intracellular levels of GAA (Tsuji et al., 1988. *J. Biochem.* 104) as do tissues from a mouse model of the same disease. Our aim was to study the lysosomal targeting of GAA and the involvement of a mannose 6-phosphate independent pathway. Previous studies have shown that GAA is synthesized as a precursor that is membrane-bound via its signal peptide (Wisselaar et al., 1993. *JBC* 268(3)). We tested whether late signal peptide cleavage is required for the lysosomal targeting of GAA. In addition, we made use of an activity-based probe that targets GAA to follow the maturation of the enzyme. Our preliminary results with HeLa cells that lack a functional mannose 6-phosphate targeting system show partial maturation of wild-type GAA. Furthermore, rapid cleavage of the signal peptide increased the solubility of GAA and impaired its lysosomal targeting. Together, these data imply an alternative endo-lysosomal sorting mechanism for GAA and show a role for the signal peptide in this process.

Preferred presentation
I have no preference

Identification and biological significance of a lysosomal glutamine transporter

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Macromolecular building blocks released by lysosomal hydrolysis are exported from lysosomes by membrane transporters. These transporters thus recycle the components of old and damaged macromolecules into cell metabolism and they restore the pools of cytosolic nutrients in the autophagic response to starvation. In some instances, lysosomal transporters also represent a cell entry route for endocytosed extracellular nutrients, such as vitamin B12.

In this study, we identify a novel lysosomal transporter specific for glutamine and asparagine, and we demonstrate its role in the nutrition and growth of Ras-activated tumor cells.

Using the transcription factor TFEB, which senses the metabolic state of lysosomes, we designed a functional assay of lysosomal amino acid storage in intact cells and used this assay to screen putative transporters of unknown or under-characterized function. Our screen identified a protein, hereafter named GlnTX, which could reverse a lysosomal overload of glutamine and asparagine, but not other amino acids, thus suggesting a role of GlnTX in the lysosomal export of these amino acids. The glutamine transport activity of GlnTX was then confirmed using radiotracer flux measurements on lysosomes purified from normal and GlnTX-deficient cells.

Cancer cells have a higher division rate and higher metabolic activity than normal cells, thus implying specific nutritional needs. In particular, glutamine is a key nutrient and a major carbon source for many cancer cells, as its metabolic transformation to glutamate and alpha-ketoglutarate replenishes the pools of tricarboxylic acid cycle intermediates. Interestingly, a recent study showed that Ras-transformed cancer cells uniquely depends on macropinocytosis uptake of extracellular proteins and their subsequent lysosomal degradation to glutamine to meet their nutritional needs and support their growth *ex vivo* and *in vivo*.

These data prompted us to examine the role of GlnTX in the growth of Ras-transformed cells.

To do this, we used Mia-PACA-2, a pancreatic adenocarcinoma-derived human cell line mutated in the KRAS gene with a strong dependence on glutamine. When glutamine is not available in the extracellular medium, these cells are able to meet their nutritional need, and grow, by macropinocytosis of extracellular serum albumin. By silencing the expression of human GlnTX, we showed that the novel lysosomal transporter is necessary for the macropinocytosis-dependent growth of Mia-PACA-2 cells.

Altogether, our study identifies a novel lysosomal amino acid transporter, GlnTX, and it shows that this transporter is a major cell entry route for the nutrition, and growth, of Ras-transformed cells by macropinocytosis of extracellular proteins. Our study thus identifies a novel protein by which cancer cells support their unique metabolic needs and suggests that nutrient starvation induced by GlnTX blockers will open the way towards developing novel anticancer therapies.

We would like to thank the “comité Ile de France” from “la Ligue nationale contre le cancer” for financial support.

Preferred presentation

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EXTL2 and EXTL3 inhibition with siRNAs as a promising substrate reduction therapy for Sanfilippo C syndrome

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Sanfilippo C syndrome, or mucopolysaccharidosis (MPS) IIIC, is an autosomal recessive lysosomal storage disorder caused by mutations in the HGSNAT gene, which encodes the enzyme acetyl-CoA α -glucosaminide N-acetyltransferase. This enzyme is involved in heparan sulfate (HS) degradation, and its deficiency leads to the storage of partially degraded HS molecules inside the lysosomes. Patients present severe and progressive neurodegeneration and no effective treatment is available as yet. Substrate reduction therapy (SRT) may be a useful option for neurological disorders of this kind and small interference RNAs (siRNAs) can be applicable to this purpose. The family of the EXTL genes play a crucial role on the synthetic pathway of HS, one of the most common glycosaminoglycans (GAGs). In particular, EXTL2 and EXTL3 are essential to initiate and terminate HS chain elongation. In this study we used four different siRNAs, two targeting the EXTL2 and two the EXTL3 gene. These siRNAs were transfected into fibroblasts of two patients in an attempt to reduce HS synthesis and storage. All the siRNAs caused a notable reduction in the mRNA levels (around 90%) of the corresponding gene and largely decreased the rates of GAG synthesis (30-60%, after 3 days) and storage (up to 24%, after 14 days). Moreover, immunocytochemistry analyses showed a clear reversion of the phenotype after treatment. Although further research is needed, the in vitro inhibition of HS synthesis genes using siRNAs presented here is a promising approach for the development of a future therapeutic option for Sanfilippo C syndrome.

Preferred presentation
I only want to present a POSTER

Identification and characterization of lysosomal calcium transporters

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Calcium is actively accumulated in endosomes and lysosomes and these acidic calcium stores play an important role in many diseases [1-5]. A multitude of Ca²⁺ transporters and channels including the TRP-ML, TPC, P2X and IP₃/ryanodine receptors have been identified [6, 7] but the precise mechanism of lysosomal Ca²⁺ uptake in mammalian cells remains unclear [1, 8].

Although a multitude of fluorescent Ca²⁺ indicators are available, measuring calcium in acidic compartments is challenging principally due to their pH-dependency [9, 10]. In order to circumvent the incompatibility of Ca²⁺ sensors with low pH, an assay using glycyl-phenylalanine-naphthylamide (GPN) has been developed [11, 12]. GPN is a membrane-permeable tripeptide that diffuses into the cell and is specifically cleaved by the lysosomal protease cathepsin C [13]. This leads to osmolytic lysis of the lysosome and released calcium can be measured in the cytosol.

By applying this method in a medium-scale siRNA screen with potential lysosomal ion transporters as target genes, we aim at identifying and characterizing novel key players important for lysosomal calcium uptake.

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Preferred presentation

I only want to present a POSTER

Targeted disruption of lysosomal α -fucosidase leads to a severe type of fucosidosis in mice

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Fucosidosis is a lysosomal storage disease that is characterized by progressive mental retardation and neurological deterioration leading to death in early childhood. It is a rare disease and until now less than 100 patients are known world-wide. Although the disease is known since 1966 there is no therapy available. The disease is caused by the deficiency of the lysosomal enzyme α -L-fucosidase, that is responsible for cleaving off fucose residues as the initial step in the degradation of complex N-glycans and glycolipids. Its deficiency leads to the accumulation of fucosylated glycoproteins and glycolipids. Here we will present the first fucosidosis mouse model that enables detailed analysis of the disease pathology.

Results: In order to understand the pathological mechanism underlying fucosidosis, we generated a constitutive knock-out mouse model by gene targeting. The complete gene knock-out was validated on genomic, transcript and protein level. Fuca1-deficient mice were viable and fertile and showed a normal appearance until the age of 6-7 months. At this age the animals became progressively inactive and especially male mice suffered from an extremely enlarged bladder. To avoid undue suffering, Fuca1-deficient mice were euthanized at 10 months of age. At the microscopic level, high amount of foam-like storage vacuoles were found in all inspected organs like liver, kidney, pancreas and spleen as well as in many cell types of the peripheral and central nervous system. In particular, loss of Purkinje cells may contribute to behavioral abnormalities observed in the Fuca1-deficient mice. Storage material was isolated from several organs, separated by TLC and analyzed by mass spectrometry. The glycopeptide Asn-GlcNAc-Fuc was identified as storage material that is also secreted into the urine of the fucosidosis mice. Conclusion: Fucosidase knock-out mice suffer from a severe form of fucosidosis leading to premature death. The phenotype closely resembles that of naturally occurring α -L-fucosidosis in human, cat and English Springer spaniel. In further studies the fucosidosis mouse model may enable the development of an enzyme replacement for the disease.

Preferred presentation

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Lysoplex: An efficient toolkit to detect DNA sequence variations in the autophagy-lysosomal pathway

Di Fruscio G^{1,2}, Schulz A³, De Cegli R¹, Savarese M², Mutarelli M¹, Parenti G^{1,4}, Banfi S^{1,2}, Braulke T³, Nigro V^{1,2}, Ballabio A^{1,4}

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The autophagy-lysosomal pathway (ALP) regulates cell homeostasis and plays a crucial role in human diseases, such as lysosomal storage disorders (LSDs) and common neurodegenerative diseases. Therefore, the identification of DNA sequence variations in genes involved in this pathway and their association with human diseases would have a significant impact on health. To this aim, we developed Lysoplex, a targeted next-generation sequencing (NGS) approach, which allowed us to obtain a uniform and accurate coding sequence coverage of a comprehensive set of 891 genes involved in lysosomal, endocytic, and autophagic pathways. Lysoplex was successfully validated on 14 different types of LSDs and then used to analyze 48 mutation-unknown patients with a clinical phenotype of neuronal ceroid lipofuscinosis (NCL), a genetically heterogeneous subtype of LSD. Lysoplex allowed us to identify pathogenic mutations in 67% of patients, most of whom had been unsuccessfully analyzed by several sequencing approaches. In addition, in 3 patients, we found potential disease-causing variants in novel NCL candidate genes. We then compared the variant detection power of Lysoplex with data derived from public whole exome sequencing (WES) efforts. On average, a 50% higher number of validated amino acid changes and truncating variations per gene were identified. Overall, we identified 61 truncating sequence variations and 488 missense variations with a high probability to cause loss of function in a total of 316 genes. Interestingly, some loss-of-function variations of genes involved in the ALP pathway were found in homozygosity in the normal population, suggesting that their role is not essential. Thus, Lysoplex provided a comprehensive catalog of sequence variants in ALP genes and allows the assessment of their relevance in cell biology as well as their contribution to human disease.

Preferred presentation

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Niemann-Pick type C diagnosis across 20 years. Analysis of the different analytical methods over time and what we have learned

Coll MJ, Macias-Vidal J, Pajares S, Arias A, Garcia-Villoria J, Ribes A
Secció Errors Congènits. Servei de Bioquímica i Genètica Molecular. Hospital Clínic, Barcelona.
CIBERER. IDIBAPS

Niemann-Pick type C (NPC) is a progressive neurodegenerative disease characterized by lysosomal/endosomal accumulation of unesterified cholesterol and glycolipids.

Here, we would like to present our experience in the laboratory since we started diagnosing NPC, 20 years ago, and also what we have learned of the different methodologies we used. Throughout these years, we have diagnosed 75 unrelated patients, 66 of them of Spanish origin.

In 1994 we set up the filipin test and in 2000, we established the molecular diagnosis for gDNA. We were able to confirm the majority of the previously biochemically diagnosed patients. Nevertheless 9 patients remained without genetic confirmation (10 unidentified alleles). This was the reason to perform the study of cDNA for all that patients. However, with this approach we only resolved 1 patient. Therefore, in 2006 we decided to treat patients fibroblasts with cycloheximide in order to avoid possible nonsense mediated mRNA decay. The results obtained, identified 9 aberrant splicing mutations resolving all the genotypes, except 1 allele, which still remains unidentified.

In 2013 we introduced in our laboratory the HPLC-ESI-MS/MS quantitation of two oxysterols in plasma, cholestane-3 β ,5 α ,6 β -triol (CT) and 7-ketocholesterol(7-KC), previously described as good biomarkers of NPC. We confirmed the increase of these metabolites in all patients for which plasma was available. The neonatal form presented with the highest levels of both oxysterols, while they were intermediate in the severe infantile form and were much lower in the late infantile, juvenile and adult form. Moreover, patients with the variant biochemical phenotype showed high CT and 7-KC, close to the levels of patients with the classical phenotype. Consequently, the method appears to be useful to identify those cases with doubtful filipin staining, including the adult presentation and the asymptomatic form of the disease as we could demonstrate in one patient. In our hands, CT is a very sensitive but not specific biomarker for the diagnosis of NPC.

All of these data enabled us to design an algorithm to achieve NPC diagnosis in our centre.

Preferred presentation

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Mucopolysaccharidoses in Tunisia: a molecular portrait of allelic heterogeneity and consanguinity

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There are 11 different enzymes involved in the stepwise degradation of glycosaminoglycans (GAGs). Deficiencies in each of those enzymes result in eight different Mucopolysaccharidoses (MPSs), all sharing a series of clinical features, though in variable degrees. Typical symptoms include organomegally, dysostosis multiplex and coarse facies. CNS, hearing, vision and cardiovascular function may also be compromised.

Traditionally, MPSs are recognized through analysis of urinary GAGs. Still, initial screenings of urinary GAGs allow discrimination between broad classes of MPSs but cannot distinguish subgroups. In fact, a definitive diagnosis may only be accessed through a combination of enzymatic assays and molecular analyses. Currently, there are countless laboratories in Europe where those biochemical and genetic tests are carried out. Nevertheless, developing countries often lack the necessary resources/expertise for proper diagnosis of rare genetic diseases. Being one of the labs where molecular genetic testing for virtually all MPSs is available for research purposes, we receive several samples from other countries, whose clinicians and/or centers struggle to get a molecular characterization of affected individuals.

Here we present our results on the molecular characterization of MPS patients we have been receiving from Tunisia. Nine families suffering from five different diseases were studied so far: 3 MPS II; 2 MPS IIIA; 2 MPS IIIB; 1 MPS IIIC and 1 MPS VI.

We have identified 9 different mutations, 5 of which were novel: 1 in the IDS gene (c.1333delC); another in the SGSH gene (p.D477N); 2 in the NAGLU gene (p.L550P and p.E153X) and yet another in the ARSB gene (p.L82P). All detected mutations were further analyzed with the most suitable approaches. Special attention was paid to the novel alterations, particularly to the missense ones, whose impact on protein structure and function was evaluated *in silico*. In general, there was a strong correlation between the observed clinical phenotype and the genotype assessed through molecular analysis. Also noteworthy is the astonishing level of homozygosity in our sample (100%), with each family presenting its own molecular defect, a pattern consistent with the occurrence of consanguineous matings in Tunisia, where such marriages are thought to provide social, economic and cultural benefits.

Altogether, our results provide a preliminary overview of the molecular basis, enzymatic defects and clinical manifestations of MPSs in Tunisia, further supporting previous reports on the high impact of inbreeding and regional endogamy on the occurrence of autosomal recessive disorders in that country. Hopefully, these results will not only contribute to improve genetic counseling for affected families, but also to highlight the need for reinforced and continuous information of general public and health professionals on the potential negative medical impact of intra-family marriages, particularly in Northern Africa, Middle East and South Asia

Preferred presentation
I have no preference.

Development of Heat Shock Protein based therapies for Lysosomal Storage Diseases

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*^aThese authors contributed equally to the work

Lysosomal storage diseases (LSDs) often manifest with severe systemic and central nervous system (CNS) symptoms. The existing treatment options are sparse and none of them are effective against the devastating neurological manifestations. Here we demonstrate proof-of-concept for heat shock protein 70 (Hsp70)-based strategies as potential pan-LSD therapies. Recombinant Hsp70 improves the binding of several sphingolipid-degrading enzymes to their essential co-factor, bis(monoacylglycero) phosphate, in vitro and reverts lysosomal pathology in primary fibroblasts from 14 patients with eight different LSDs. It penetrates effectively to murine tissues including CNS, inhibits glycosphingolipid accumulation in murine models of Fabry (GLA^{-/-}), Sandhoff (HEXB^{-/-}) and Niemann-Pick type C (NPC1^{-/-}) diseases, and effectively alleviates a wide spectrum of disease-associated neurological symptoms in HEXB^{-/-} and NPC1^{-/-} mice. Importantly, oral administration of arimoclomol, a clinically enabled small molecule co-inducer of Hsp70, recapitulates the pan-LSD and neurological potential of recombinant Hsp70 encouraging the development of Hsp70-based therapies for LSDs.

Preferred presentation

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The two tales of glycosphingolipids in Natural Killer T lymphocyte activation

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Invariant Natural Killer T (iNKT) cells are innate-like T lymphocytes that express a semi-invariant TCR and are activated by lipid antigens presented by CD1d molecules. Several glycosphingolipids, including α -Galactosylceramide (α -GalCer), are able to activate iNKT cells. However little is known whether the accumulation of CD1d-binding and non-stimulatory glycosphingolipids interfere with iNKT cell activation.

Numerical and functional defects of iNKT cells have been described in sphingolipidoses including Fabry disease (accumulation of globotriaosylceramide, Gb3). The mechanisms responsible for iNKT cell alterations in this disease are not well understood.

Here, we analyzed the effect of Gb3 on CD1d-mediated iNKT cell activation in vitro using human cells and in vivo in the mouse. We found that Gb3 can negatively modulate iNKT cell activation by competing with endogenous and exogenous lipid antigens for CD1d binding. Monocytes or immobilized CD1d incubated simultaneously with Gb3 and α -GalCer induced a weaker iNKT cell response as compared to incubation with α -GalCer alone. This effect was also applicable to endogenous antigens, as loading of antigen-presenting cells with Gb3 reduced iNKT cell autoreactivity. We confirmed that Gb3 was competing with α -GalCer for CD1d binding by finding a reduction in the formation of CD1d: α -GalCer complexes in the presence of Gb3. This competition effect was also observed in vivo when iNKT cells were stimulated by dendritic cells loaded with both Gb3 and α -GalCer.

The presence of high amounts of Gb3 in Fabry disease might contribute to reduced iNKT cell stimulation and proliferation, thus explaining iNKT cell numerical and functional defects.

The analysis of iNKT cells on sphingolipidoses highlights the complexity of iNKT cell activation and the importance of non-antigenic glycosphingolipids in the modulation of this process.

Preferred presentation

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Analysis of circulating and tissue-specific micro-rnas in pompe disease

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Pompe Disease (PD) is a metabolic myopathy caused by the deficiency of the lysosomal hydrolase acid-alpha-glucosidase (GAA). GAA deficiency results in generalized tissue glycogen accumulation and secondary cardiac and skeletal muscle destruction. At present enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA), is the only available therapeutic approach to PD. Despite progress in treatment, PD remains associated with unmet medical needs, such as variability in clinical outcome in response to ERT, lack of markers of disease progression and markers of ERT efficacy, need for new therapeutic targets.

Aim of the study: to identify “disease-specific” differentially expressed miRNAs (DE-miRNAs) as new biomarkers for PD and as tools to follow disease progression and therapeutic efficacy.

Methods: miRNA profiling was performed by next-generation sequencing and bioinformatic analysis in samples (plasma, heart and gastrocnemius) from PD mice. Samples were obtained at two stages of disease progression (3 and 9 months). For each time-point 3 PD animals were analyzed and compared with age-matched wild-type animals. miRNA analysis in plasma from 17 PD patients was performed by RT-PCR.

Results: we found 1 DE-miRNA in plasma (miR-486) at 9 months with statistical significance (FDR < 0.05). We also found 219 DE-miRNAs (FDR < 0.05) in muscle (gastrocnemius), and 35 DE-miRNAs in heart. In total, 104 miRNAs were differentially expressed at 3 months, 109 at 9 months, 42 were differentially expressed at both ages. Some of the DE-miRNAs identified in tissues are already known to modulate the expression of genes involved in pathways such as autophagy, muscle regeneration, inflammation that are relevant for PD pathophysiology.

In plasma samples from 7 infantile-onset PD patients miR-486 levels were higher than in age-matched controls. In samples from 10 late-onset, average miR-486 levels were also higher than in controls.

Conclusions: we have identified circulating and tissue-specific DE-miRNAs that may represent new/valuable biomarkers to monitor disease progression. The plasma levels of miR-486 appear to correlate with disease severity.

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Preferred presentation

I only want to present a POSTER.

A physiological role of TFEB in LSDs

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Lysosomal storage diseases (LSDs) are characterized by the accumulation of storage material, due to defective lysosomal degradation and impaired cellular clearance in both the central nervous system and in visceral tissues. Recently, we described TFEB as master regulator of lysosomal function. In normal conditions TFEB is located in the cytoplasm, thus being transcriptionally inactive. Cytoplasm-to-nucleus translocation of TFEB results in its activation. Several studies have demonstrated that overexpression of TFEB by viral-mediated gene transfer results in efficient cellular clearance in murine models of LSDs. The aim of this study was to investigate the physiological role of TFEB in LSDs by using both *in vitro* and *in vivo* approaches. Initially, we characterized the expression and the subcellular localization of endogenous TFEB in several mouse models of neurodegenerative LSDs (i.e. MPSIIIa, MSD, and Sandhoff disease) and found that TFEB transcript was up-regulated in neurons and microglial cells and that TFEB protein had a predominantly nuclear localization in these tissues, suggesting the presence of an activation of TFEB function as a response to the disease process. In order to better understand the physiological role of TFEB in LSDs, we have focused our attention on Gaucher disease (GD), whose phenotype can be mimicked in mice by pharmacological treatment. GD is a rare autosomal recessive disorder caused by the defective activity of the lysosomal hydrolase β -glucocerebrosidase (GBA). The defective enzyme leads to an accumulation of its substrate, gluco-cerebroside, in mononuclear phagocytes. We generated chemically induced *in vivo* and *in vitro* models that recapitulate the disease by using Condurotol-B-epoxide (CBE), which is a highly specific inhibitor of the GBA enzyme. We found that in HeLa cells, GBA deficiency leads to TFEB nuclear translocation. As observed for other LSDs, TFEB overexpression in cells and tissues from CBE-treated mice resulted in the disappearance of lysosomal storage, as measured by the clearance of accumulated gluco-cerebroside. The opposite situation was observed in TFEB loss-of-function studies. In both CBE-treated HeLa cells carrying CRISPR-Cas9-mediated deletion of TFEB and in the liver of CBE-treated TFEB liver-specific conditional KO mice we observed a very significant increase in lipid accumulation and an enlargement of the lysosomal compartment compared to CBE-treated wild type cells and mice. These data suggest a protective role of TFEB in LSDs, which is likely to represent a physiological attempt to promote cellular clearance in situations associated with lysosomal storage.

Preferred presentation

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