ESGLD 2017

European Study Group on Lysosomal Diseases

21ST ESGLD WORKSHOP AND GRADUATE COURSE

ECULLY (LYON), FRANCE

September 13th - 17th 2017

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21st ESGLD WORKSHOP (2017)

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Short Course on Lysosomes and Lysosomal Diseases

For Graduate Students and Young PostDocs

Wednesday, September 13th

Basic Aspects of Lysosomal Biology

14:00 **Introductory remarks**
Bruno Gasnier and Marie T Vanier

14:15 – 15:00 **Thomas Braulke**
(Universitätsklinikum Hamburg-Eppendorf, Germany)
Biogenesis of lysosomes

15:00 – 15:45 **Carmine Settembre**
(Telethon Institute of Genetics and Medicine, Pozzuoli, Italy)
Autophagy and lysosomal adaptation

15:45 – 16:15 ***Coffee Break***

16:15 – 17:00 **Paul Saftig**
(Kiel University, Germany)
Lysosomal membrane proteins

17:00 – 17:45 **Bruno Gasnier**
(Paris Descartes University, France)
Lysosomal channels and transporters

17:45-18:15 **Designation of flash-talk discussion leaders**

19:30 **Dinner**

Short Course on Lysosomes and Lysosomal Diseases, continued..

Thursday, September 14th morning

Translational and Clinical Aspects

8:45 – 9:30 **Timothy Cox**
(University of Cambridge, UK)
Lipid degradation and lipid storage diseases

9:30 – 10:15 **Marie T. Vanier**
(Inserm, Lyon, France)
Niemann Pick C disease

10:15 – 10:45 ***Coffee Break***

10:45 – 11:30 **Angela Schulz**
(Universitätsklinikum Hamburg-Eppendorf, Germany)
Pathology and treatment of neuronal ceroid lipofuscinoses

11:30 – 12:15 **Brian Bigger**
(University of Manchester, UK)
Therapy of lysosomal storage diseases

12:30 – 14:00 **Lunch**

Thursday, September 14th, afternoon

Flash Talks from Graduate Students and Postdocs

14:00-16:30 3-min max. 3-slide talks by trainees to present their research or clinical study

16:30-16:45 Flash talk 'awards'

21st ESGLD WORKSHOP

Ecully (Lyon), France

14-17 September, 2017

Scientific Programme

Thursday, September 14th

18-15 **Opening – Welcome address**

18.30 – 19.30 **Keynote lecture 1** – introduced by **Jérôme Ausseil** (Amiens)

Marc Tardieu (Université Paris-Sud) – ***Gene therapy in lysosomal diseases: the example of Mucopolysaccharidosis type IIIB***

19.30 **Get-together and Dinner**

Friday, September 15th

8.30 – 11.40 Session 1: Molecular/cellular and pharmacological studies

Discussion leaders: **Jérôme Ausseil** (Amiens) and **Thomas Braulke** (Hamburg)
(15-min talks + 5-min questions)

8:30 **O-1**	**Pshezhetsky A** Montreal	Inhibitors of lysosomal neuraminidases 1 and 3 as potential candidates for treating atherosclerosis
8:50 **O-2**	**Matzner U** Bonn	Genetically engineered arylsulfatase A with increased catalytic rate for enzyme-based therapies of metachromatic leukodystrophy
9:10 **O-3**	**Anne C** Paris	Structure-based designed inhibitors of sialin as potential scaffolds for pharmacological chaperone treatment of Salla disease
9:30 **O-4**	**Pan X** Montreal	Chaperone therapy for mucopolysaccharidosis type IIIC
9:50	***Coffee Break***	
10:20 **O-5**	**Boonen M** Namur	Spastic paraplegia 21: a lysosomal disease?
10:40 **O-6**	**van der Lienden MJC** Leiden	HEPES drives a MiT/TFE-mediated lysosomal-autophagic gene network in cultured cells: a call for caution
11:00 **O-7**	**Winter D** Bonn	Mass spectrometry based targeted quantification of the lysosomal proteome by stable isotope labeled concatenated proteins
11:20 **O-8**	**Snanoudj-Verber S** Chapel Hill, Paris	AAV9-based gene therapy restores enzymatic activity in a mouse model for aspartylglucosaminuria

11:40 – 12:30 **ESGLD General Assembly** **(for ESGLD members only)**

Friday, September 15th, continued…

12.30 – 14.00 **Lunch**

14.00 – 17.30 **Networking**

18.00 – 19.40 **Session 2: Lysosomal channels and transporters**

Discussion leader: **Paul Saftig** (Kiel)
(15-min talks + 5-min questions)

18:00	**Lloyd-Evans E**		The Batten disease protein CLN3 is a lysosomal ion
O-9		Cardiff	channel that regulates lysosomal response to swelling
18:20	**Waller-Evans H**		Inhibition of NAADP mediated lysosomal Ca^{2+} signalling
O-10		Cardiff	induces Niemann-Pick type C phenotypes in cells and animal models
18:40	**Froese DS**		Mutations in ABCD4 disrupt interaction between the
O-11		Zurich	lysosomal proteins ABCD4 and LMBD1 involved in vitamin B12-trafficking
19:00	**Terres Y**		Synthetic lethality between two lysosomal amino acid
O-12		Paris	transporters
19:20	**Buttgereit A**		Knock-in mouse reveals importance of slow gating of
O-13		Berlin	lysosomal H+/Cl- exchange in lysosomal function and bone resorption

19:40 End of Session

20.00 **Dinner**

…/…

Saturday September 16th

8.30 – 12.30 Session 3: Pathophysiology and disease models

Discussion leaders: **Volkmar Gieselmann** (Bonn) and **Carmine Settembre** (Naples)
(15-min talks + 5-min questions)

8:30 **O-14**	**Raimundo N** Göttingen	Lysosomal malfunction impairs mitochondria via a transcriptional mechanism
8:50 **O-15**	**Monfregola J** Naples	Generation of a lysosomal storage disorder CRISPr biobank for the study of lysosomal storage disorders
9:10 **O-16**	**Linhorst A** Bielefeld	The lysosomal hydrolase Plbd2 – From knockout to functional role
9:30 **O-17**	**Trabszo C** Bielefeld	Arylsulfatase K (ARSK) – The missing link in glycosaminoglycan degradation. Arsk-knockout mouse characterization
9:50 **O-18**	**Di Lorenzo G** Hamburg	Role of the gamma-subunit of GlcNAc-1-phosphotransferase in the pathogenesis of mucolipidosis type III
10:10	***Coffee break***	
10:50 **O-19**	**Cabasso O** Tel-Aviv	The fruit fly drosophila melanogaster as a model system to study Gaucher disease
11:10 **O-20**	**Maor G** Tel-Aviv	The contribution of mutant glucocerebrosidase to the aggregation of alpha synuclein
11:30 **O-21**	**Schaaf GJ** Rotterdam	Progressive lysosomal dysfunctioning inactivates muscle stem cells and blocks muscle regeneration in Pompe disease
11:50 **O-22**	**Parker H** Manchester	Mucopolysaccharidosis IIIA storage substrate drives an innate immune neuro-inflammatory response
12:10 **O-23**	**Settembre C** Naples	mTORC1 hyperactivation arrests bone growth in lysosomal storage disorders
12:30	End of Session	

12.30 – 14.00 **Lunch**
(meeting of the ESGLD board, and lunch, in a dedicated room)

Saturday September 16th continued…

14.00 – 16.20 Session 4: Diagnostics and clinical studies

Discussion leaders: **Thierry Levade** (Toulouse) and **Angela Schulz** (Hamburg)
(15-min talks + 5-min questions)

14:00 **O-24**	**Pettazzoni M** Lyon	Multiplex LC-MS/MS lysosphingolipids analysis in plasma for the screening of sphingolipidoses and Niemann-Pick disease type C
14:20 **O-25**	**Ferraz MJ** Leiden	Glycosphingoid bases (lyso-glycosphingolipids) in lysosomal storage disorders
14:40 **O-26**	**Zhang K** Cambridge MA	Evaluation of glucosylsphingosine as a biomarker of the Eliglustat treatment -response in patients with Gaucher disease Type 1 (GD1)
15:00	***Coffee Break***	
15:20 **O-27**	**Tebani A** Rouen	Urinary metabolomics and data modeling unveil mucopolysaccharidosis type I metabolic impairments
15:40 **O-28**	**Arends M** Amsterdam	Retrospective study of long-term outcomes of enzyme replacement therapy in Fabry disease: analysis of prognostic factors
16:00 **O-29**	**Morand O** Allschwill	Lucerastat, an iminosugar for substrate reduction therapy: safety, tolerability, PD and PK in adult subjects with Fabry disease

16:20 End of session

16:20 -18:00 Attended Poster Session

18:15 – 19:15 Keynote lecture 2 – introduced by **Bruno Gasnier** (Paris)

Haoxing Xu (University of Michigan) – ***Ion channels in the lysosome: opening the gate to the cell's recycling center***

20:00 **ESGLD dinner**

Sunday September 17th

8.30 – 12.20 Session 5: Gene and cell therapy approaches

Discussion leaders: **Catherine Caillaud** (Paris) and **Brian Bigger** (Manchester)
(15-min talks + 5-min questions)

8:30 **O-30**	**Bigger BW** Manchester	A BBB crossing peptide with lentiviral-mediated stem cell gene therapy fully corrects Mucopolysaccharidosis II
8:50 **O-31**	**Ferla R** Naples	Combination of gene and enzyme replacement therapies for mucopolysaccharidosis type VI
9:10 **O-32**	**Rouvière L** Paris	AAV9 gene transfer in Sandhoff mice: correction of brain and cerebellum using a combined way of administration
9:30 **O-33**	**O'Leary C** Manchester	Correction of neurological manifestations of MPSIIIC by a novel rationally designed neurotropic AAV gene therapy vector
9:50 **O-34**	**Peruzzo P** Udine	RNA based therapies for glycogenosis type II due to the common c.-32-13T>G mutation
10:10	***Coffee Break***	
10:40 **O-35**	**Azario I** Monza	Neonatal umbilical cord blood transplantation halts disease progression in the murine model of MPS-I
11:00 **O-36**	**Liao A** Manchester	Non-depleting anti-CD4 monoclonal antibody induces immune tolerance to enzyme replacement therapy in a mucopolysaccharidosis type I mouse model
11:20 **O-37**	**Pijnappel P** Rotterdam	Immune Tolerance Induction by Lentiviral Stem Cell Gene Therapy in Pompe Disease
11:40 **O-38**	**Mitchell NL** Christchurch	AAV gene transfer halts disease progression in clinically affected sheep with CLN5 Batten disease
12:00 **O-39**	**Mole SE** London	BATCure: An H2020 Consortium developing new therapies for Batten disease
12:20		**Closing remarks**
12:35		**Young investigator's award**
13:00		**Lunch-box and departure**

POSTER SESSION (by alphabetic order of first author)

First Author	Presenter	Poster #	Title
Alshehri AS	Alshehri AS	**P-1**	Simple fluorimetric test for lysosomal swelling as a means to identify, monitor and develop therapies for all lysosomal storage diseases
Arash-Kaps L	Hennermann JB	**P-2**	The broad clinical variability of GM1 gangliosidosis
Badell-Grau RA	Badell-Grau RA	**P-3**	Characterising the fundamental cell biology of CLN8 disease for the purpose of drug screening and development
Benetó N	Benetó N	**P-4**	*EXTL2* as a target for substrate reduction therapy in iPSC-derived neurons from Sanfilippo C patients
Boer D	Boer D	**P-5**	Activity of lysosomal glucocerebrosidase towards xylosides
Calcagnì A	Calcagnì A	**P-6**	A cellular model of Neuronal Ceroid-Lipofuscinosis type 3 created by CRISPR-Cas9 provides new insights into disease pathogenesis
Carpenter K	Priestman DA	**P-7**	Circulating glycosphingolipids in patients with GM2 gangliosidosis
Castillo O	Coll MJ	**P-8**	Identification of disorders of glycoprotein degradation and other related diseases using a new HPLC method
Ciana G	Ciana G	**P-9**	Effects of high-oral ambroxol chaperone therapy in two Italian patients with type 3 Gaucher disease
Ciana G	Ciana G	**P-10**	Very long-term bone mineral density response in a cohort of Gaucher patients treated with ERT from childhood to adulthood

Coutinho MF	Coutinho MF	**P-11**	Genetically modulated Substrate Reduction Therapy for Mucopolysaccharidoses – in vitro studies
Dardis A	Dardis A	**P-12**	Niemann Pick type C in Italy: an update of molecular and biochemical data
Darwiche W	Darwiche W	**P-13**	Among accumulated Heparan sulfate oligosaccharides, hexasaccharides are the most pathogenic fractions involved in glia activation in Sanfilippo syndrome
Dubot P	Dubot P	**P-14**	Early hematopoietic stem cell transplantation in a MPS type VII boy
Dubot P	Dubot P	**P-15**	Is acid glucosylceramidase a player in the development of cutaneous melanoma?
Ferri L	Ferri L	**P-16**	Newborn screening for Fabry disease in the Italian regions of Tuscany and Umbria: current overview
Hřebíček M	Hřebíček M	**P-17**	Strategies compared: diagnostic next generation sequencing (NGS) and biochemical markers of NPC in at risk populations
Kaade E	Kaade E	**P-18**	Investigation of the lysosomal proteome in different nutrient conditions
Korolenko TA	Korolenko TA	**P-19**	Chitotriosidase activity and expression in mice with lipid storage syndrome treated by macrophage stimulator
Kytidou K	Kytidou K	**P-20**	Activity-based labeling and detection of active lysosomal glycosidases: application in diagnostic screening of urine samples
Matos S	Alves S	**P-21**	Development of an antisense-mediated exon skipping approach as a therapeutic option for the ML II-causing mutation c.3503_3504delTC

Mauhin W		**P-22**	Anti-agalsidase antibodies associated with renal transplantation in Fabry disease
Monaco A	Monaco A	**P-23**	Treating neuronal proteostasis in lysosomal storage diseases
Nelvagal HR	Nelvagal HR	**P-24**	Early onset gait abnormalities and spinal cord pathology in a mouse model of CLN1 Disease
Paciotti S	Beccari T	**P-25**	CSF lysosomal enzymes activity and GBA1 genotyping in Parkinson's disease
Palmer DN	Palmer DN	**P-26**	Cross–regulation of *CLN5* and *CLN6* gene expression in ovine Batten disease models
Pettazzoni M	Pettazzoni M	**P-27**	Plasmatic biomarkers for the screening of Niemann-Pick type C disease: experience in a clinical setting in France
Pupyshev AB	Pupyshev AB	**P-28**	Suppressed autophagy in a mouse model of neurodegeneration and autophagy stimulation in brain by rapamycin and trehalose
Rigon L	Rigon L	**P-29**	Glycosaminoglycan profile in the Mucopolysaccharidosis type II mouse model at baseline and after 6 weeks treatment with ERT
Rodriguez CE	Rodriguez CE	**P-30**	Enzymatic method for the determination of the non-lysosomal glucosylceramidase
Rudnik S	Rudnik S	**P-31**	Characterisation of the phosphatidylinositol(4,5)bisphosphate 4-phosphatase TMEM55A and TMEM55B
Ruiz-Andres C	Gort L	**P-32**	Lysosomal acid lipase deficiency in 23 Spanish patients: High frequency of the novel c.966+2T>G mutation in Wolman disease

Russell KN	Russell KN	**P-33**	Longitudinal *in vivo* monitoring of disease progression and viral mediated gene injection therapy in ovine Batten disease
Rybova V	Asfaw B	**P-34**	Model of CNS involvement for mucopolysaccharidosis type II: neural cells from induced pluripotent stem cells of a patient
Sechi A	Bembi B	**P-35**	Successful desensitization to enzyme replacement therapy by using omalizumab in a patient with late-onset Pompe disease
Sudrié-Arnaud B	Sudrié-Arnaud B	**P-36**	Metabolic causes of non immune hydrops fetalis: next generation sequencing panel as first line investigation
Sudrié-Arnaud B	Sudrié-Arnaud B	**P-37**	Next generation sequencing strategy for lysosomal storage diseases diagnosis
Tomanin R	Tomanin R	**P-38**	Mucopolysaccharidosis type VI (MPS VI) and molecular analysis: A review of published classified variants in the *ARSB* gene
Van der Wal E	Bergsma AJ	**P-39**	Antisense based correction of *GAA* splicing in iPSC-derived skeletal muscle cells from Pompe patients that carry the IVS1 variant
Waller-Evans H	Waller-Evans H	**P-40**	Lysosomal dysfunction in Smith-Lemli-Opitz syndrome caused by inhibition of the NPC1 protein can be corrected using some NPC therapies
Zanetti A	Tomanin R	**P-41**	A targeted sequencing panel for the analysis of exons and conserved intronic sequences of 50 LSD genes
Zech I	Gieselmann V	**P-42**	Developing substrate reduction therapy for metachromatic leukodystrophy

Abstracts of Oral Communications

(by order of the programme)

O-1

Inhibitors of lysosomal neuraminidases 1 and 3 as potential candidates for treating atherosclerosis

Smutova V[1], Fougerat A[1], Demina E[1], Guo T[2], Zou C[2], Miyagi T[3], Laffargue M[4], Cairo CW[2], and Pshezhetsky AV[1]

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Atherosclerosis is a chronic vascular disease characterized by lipid retention and inflammation of the vessel wall. The disease starts from the uptake by resident macrophages of atherogenic modified low-density lipoproteins (LDL) resulting in formation of arterial fatty streaks and eventually atheromatous plaques. Increased plasma sialic acid levels or reduced sialylation of LDL and aortic endothelium have been associated previously with atherosclerosis and coronary artery disease in human patients but the mechanism underlying this association has been never explored. In the current study we investigated the hypothesis that lysosomal neuraminidases also present on the surface of hematopoietic cells and/or arterial endothelium contribute to development of atherosclerosis by removing sialyc acid residues from glycan chains of LDL glycoproteins and glycolipids. Our results demonstrate that in vitro desialylation of a major LDL glycoprotein – Apolipoprotein B 100 (ApoB) by human neuraminidases 1 and 3 increases the uptake of human LDL by cultured human macrophages, but not by hepatocytes. It also leads to increased accumulation of LDL in the aortic wall of mice. We further show that in the murine model of atherosclerosis, Apolipoprotein E (ApoE) knockout mice, genetic deficiency of neuraminidases 1 and 3 or treatment of mice with specific inhibitors of these enzymes significantly delays formation of fatty streaks in the aortic root without affecting the plasma cholesterol and LDL levels. Together, our results suggest that neuraminidases 1 and 3 trigger the initial phase of atherosclerosis, formation of aortic fatty streaks by reducing sialylation of LDL and increasing their uptake rate.

O-2

Genetically engineered arylsulfatase A with increased catalytic rate for enzyme-based therapies of metachromatic leukodystrophy

Matzner U[1], Gieselmann V[1]

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Functional deficiencies of the lysosomal enzyme arylsulfatase A (ASA) cause the lysosomal storage disease metachromatic leukodystrophy (MLD). MLD is characterized by progressive demyelination, deteriorating neurological symptoms and early death. Preclinical studies of enzyme replacement therapy (ERT) and gene therapy (GT) in MLD mouse models were promissing and led to phase I/II clinical trials. Benefit of these treatments is limited by the blood-brain barrier preventing efficient transfer of enzyme from the blood circulation to the brain parenchyma (ERT) and a low number of transduced ASA-producer cells (GT), respectively. Elevation of the ASA activity in the CNS, therefore, remains the main challenge in optimizing enzyme-based therapies of MLD. Using a rationale design approach we addressed this issue by improving the catalytic rate constant of the therapeutic enzyme. The optimization strategy was based on the observation that murine ASA has a 5.9-fold higher catalytic rate compared to its human orthologue. We used site-directed mutagenesis to substitute amino acids of the human ASA polypeptide by their murine homologue. Subsequently, CHO-K1 cells were transiently transfected and the specific activities of the chimeric ASAs were measured in the conditioned media. By exchanging amino acids individually and in combination, the three activity-promoting exchanges M202V, T286L and R291N could be identified. The three positions are located apart from the active site in two non-adjacent polypeptide regions of unknown function. Compared to wildtype human ASA, ASA-M202V and ASA-M202V,T286L,R291N exhibit 3.4- and 4.7-fold higher turnover numbers, respectively. The two ASA-mutants might be valuable tools to increase therapeutic efficacy and diminish side effects in future ERT and GT applications.

O-3

Structure-based designed inhibitors of sialin as potential scaffolds for pharmacological chaperone treatment of Salla disease

Dubois L[1], McCort-Tranchepain I[1], Chausset-Boissarie L[1], Debacker C[2], Sagné C[2], Ribes C[2], Acher F[1], Gasnier B[2]*, Pietrancosta N[1]*, Anne C [2]* **equal contributions*

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Free sialic acid cleaved off from glycoproteins and glycolipids degraded in lysosomes are exported to the cytosol by sialin, the lysosomal sialic acid exporter encoded by the *SLC17A5* gene. Sialin is defective in two inherited sialic acid storage diseases: infantile sialic acid storage disease (ISSD) and Salla disease. ISSD is an early-lethal, multisystemic disease caused by diverse *SLC17A5* loss-of-function mutations, whereas Salla disease is a progressive, non-lethal neurological disease almost exclusively associated with the missense, hypomorphic mutation R39C. The accumulation of free sialic acid in patients' cells is a hallmark of both diseases. There is no specific treatment.

In Salla disease, pharmacological chaperones might be a therapeutic option as the impairment of lysosomal sialic export by the R39C mutation results from two partial effects: on one hand, the transport activity is decreased by ~70% and, on the other hand, the protein only partially reaches the lysosome, presumably because of misfolding *(Morin et al (2004) EMBO J 23:4560-4570)*. High-affinity ligands of the sialic acid-binding site might thus promote correct folding of the R39C mutant, rescue its lysosomal localization and thereby increase lysosomal sialic acid export to ~30% of the wild-type level, a value close to that (50%) of healthy heterozygous mutation carriers. In this study, we aimed at identifying such ligands as a first step towards a pharmacological chaperone approach of Salla disease.

To address this issue, we built a 3D homology model of human sialin and validated the sialic acid-binding site by site-directed mutagenesis and by proof-of-principle virtual high-throughput drug screening (vHTS) *(Pietrancosta et al (2012) J Biol Chem 287:11489-11497)*. We next performed a vHTS of 80,000 commercially available compounds on this 3D model and, after wet lab validation, identified diverse ligands with a >100-fold higher affinity than the natural substrate N-acetylneuraminic acid. Optimization of these hits by analogue synthesis and structure-activity relationship studies further increased the affinity.

Inhibitors with sub-µM affinity were then characterized in competition experiments. Surprisingly, some of them apparently acted as non-competitive inhibitors despite their initial identification as ligands of the sialic acid-binding site, possibly because they are permeant and bind to both outward-open and inward-open conformations of the transporter.

The best cell-permeant ligand was then tested for its capacity to rescue the intracellular localization defect of the R39C mutant associated with Salla disease. Incubating cells expressing R39C sialin with this compound rescued lysosomal localization. The effect of this compound on free sialic acid storage in patient fibroblasts is in progress.

O-4

Chaperone therapy for Mucopolysaccharidosis type IIIC

Pan X[1], Héon-Roberts R[1], Li S-G[2], Wu HY[1], De Britto Pará De Aragão C[1], Ekins S[3], Freundlich JS[2], Boyd R[4], Pshezhetsky AV[1]

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Mucopolysaccharidosis type IIIC (MPS IIIC) is an autosomal recessive lysosomal storage disorder that manifests with sleep disorders, behavioural problems, and progressive dementia in children, leading to death by the second decade of life in most patients [1]. The disease is caused by mutations in the gene encoding for heparan sulphate acetyl-CoA: α-glucosaminide N-acetyltransferase (HGSNAT), which is involved in lysosomal catabolism of heparan sulphate (HS). About 50% of patients are affected with missense HGSNAT mutations, which prevent correct folding of the enzyme and cause its retention in the endoplasmic reticulum and degradation in proteasomes [2]. Such mutants can be rescued by pharmaceutical chaperones, which would bind to the enzyme in the active conformation, increasing its chance of folding properly. MPSIIIC is an excellent candidate for chaperone therapy because a threshold activity of ~15% is sufficient to prevent severe pathology [3]. Besides, >50% of patients are affected with missense mutations interfering with the folding of the enzyme.

We have synthesized several iminosugar analogues of the natural HGSNAT substrate, and tested them for their ability to inhibit recombinant human HGSNAT and increase the residual activity of mutant HGSNAT in the cultured cells of MPSIIIC patients. Several compounds were specific HGSNAT inhibitors with the best having a K_i in 10-μM range and showed chaperone activity in cultured MPSIIIC fibroblasts.

To test efficacy of chaperones *in vivo,* we generated a novel knock-in mouse model of MPS IIIC mouse model carrying the frequent human HGSNAT mutation, P283L, which causes enzyme missfolding [2]. The C57Bl6 mouse heterozygous for the P283L substitution in the *Hgsnat* gene was generated by CRISPR-Cas9 technique. The specific activity of HGSNAT and control lysosomal enzymes were measured in the brain, liver, kidney, and lung of 2 and 4-month-old wild type, $HGSNAT^{P283L}$, and previously described HGSNAT knockout mice[4]. Brain tissues were analyzed for the markers of astrogliosis, neuroinflammation, and lysosomal storage. Our preliminary results show significantly decreased activity of the HGSNAT enzyme (2-4% of the wild-type control) in the tissues of $HGSNAT^{P283L}$ mice, similar to that measured in the knockout mice. On the contrary, the marker of lysosomal accumulation, beta-hexosaminidase activity showed a significant increase as compared with the wild-type controls as well as with the knockout mice in the tissues measured, consistent with the dominant-negative effect of the missfolded HGSNAT mutant.

We will continue to test the chaperone candidates in MEF cells of $HGSNAT^{P283L}$ mice with the goal of identifying compounds capable of boosting the residual HGSNAT level to >15% of normal in the mice themselves. Taken together, the data collected from

these experiments will be valuable in the progression towards finding a treatment for MPS IIIC and related neuropathies.

References

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2. Feldhammer M et al (2009) PLoS ONE 4(10): e7434.
3. Haer-Wigman L et al (2015) Hum Mol Genet 24:3742-51.
4. Martins C et al (2015) Brain 138: 336-355.

O-5

Spastic Paraplegia 21: a lysosomal disease?

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Hereditary Spastic ParapleGia (SPG) 21 is an autosomal recessive disorder caused by mutations in the gene coding for Maspardin, a 35 kDa soluble protein. Progressive lower limb spasticity due to axonal degeneration of corticospinal neurons and dementia are the main manifestations of this disease. Yet, the underlying molecular causes remain elusive, as the function of Maspardin has not been elucidated. It has been reported that Maspardin localizes to the endosomes, trans-Golgi Network and cytosol in HeLa cells. However, our recent findings indicate that a large part of the membrane-associated Maspardin population of rat liver cells resides on lysosomes. To elucidate precisely the site where Maspardin localizes, and possibly uncover where this protein exercises its function, we investigated its subcellular localization in several cell lines (HeLa, human hepatocytes HEP3B and human neuroblastoma cells SK-N-BE) using a combination of subcellular fractionation and immunofluorescence methods. Interestingly, our results demonstrate that Maspardin exhibits a higher enrichment in lysosome-containing fractions than VPS26, a component of the retromer complex that is recruited on the endosomes. Moreover, we identified a four amino acid motif required for the association of Maspardin with the lysosome, which led us to conclude that Maspardin is a lysosome-associated protein recruited from the cytosol. An intriguing observation is that the population of membrane-associated Maspardin proteins was consistently lower in HeLa and SK-N-BE cells compared to HEP3B cells, raising the possibility that the cellular process to which Maspardin contributes might not be similarly active in all cell types under basal conditions. According to the recently published subcellular localization map that we created with Drs. P.Lobel, D. Sleat and colleagues (Rutgers Biomedical and Health Sciences, NJ, USA) by combining analytical subcellular fractionation to quantitative mass spectrometry, Maspardin and several other SPG-associated proteins share a very similar intracellular distribution in the rat liver (prolocate.cabm.rutgers.edu; Jadot et al 2017, MCP 16: 194-212). As lysosomal abnormalities have been documented in several of these SPG cases, it is worth considering that SPG21 may be an uncharacterized lysosomal disease. Several lines of research are currently ongoing in our lab to test this hypothesis.

O-6

HEPES Drives a MiT/TFE-mediated Lysosomal-Autophagic Gene Network in Cultured Cells: A Call for Caution

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The lysosome has been increasingly acknowledged as a crucial organelle in nutrient-sensing and cell metabolism. Through mTORC1 dependent regulation of the mircophtalmia-transcription factor E (Mi/TFE) family, the lysosome serves as a metabolic signaling hub that integrates metabolic demand and nutrient availability into a coordinated cellular response. Despite the fact that this organelle is known to be important in cellular metabolism and signaling, the impact of conventional culturing methods on lysosomal integrity and the subsequent signaling has not been fully appreciated. In this study we show that HEPES, a buffering compound in mammalian cell culture, potently induces lysosomal biogenesis. By abolishing the cytosolic retention of the MiT/TFE family members TFEB, TFE3 and MITF, HEPES drives the expression of autophagic-lysosomal and host-immune genes, thereby changing proteolytic capacity, autophagic flux and immune reactivity of cultured cells. gpNMB, a glycoprotein which expression is dramatically induced upon lysosomal stress and serves as a biomarker in lysosomal storage diseases, was found among the most upregulated proteins upon HEPES addition. Its elevated expression was found to be dependent on TFEB, TFE3 as well as MITF. The MiT/TFE driven phenotype appears to be independent of mTORC1. Our data highlight cautionary use of chemical buffering agents in culture media, especially in the context of lysosomal disease diagnostics, due to their potentially confounding effects on the basal state of lysosomes and cellular metabolism.

O-7

Mass Spectrometry based Targeted Quantification of the Lysosomal Proteome by Stable Isotope Labeled Concatenated Proteins

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Studies investigating lysosomal function typically focus on a single protein or a representative subset of lysosomal proteins dependent on their ease of detection in Western blot or qPCR. Therefore, out of the ~150 proteins confirmed to be of lysosomal localization, only a small subset can be analyzed under different conditions and their abundance is usually used as surrogate for the whole lysosome. Data representing the lysosome, and therefore all of its proteins, as an entity do not exist. Therefore the quantitative composition of lysosomes in different cell types or conditions and whether this composition is static or dynamic remains completely unclear.

Several studies suggest that the composition of lysosomes is dynamic with respect to the metabolic situation of the cell, the type of cell (or tissue) and whether the cell is healthy or mechanisms affecting the lysosome are impaired. In order to be able to investigate this, we developed a strategy based on synthetic standard proteins termed QConCats (Pratt et al. Nat. Prot., 2006), allowing to quantify the absolute amount of 144 lysosomal proteins concomitantly from any sample of mouse origin. The underlying principle is, that we selected representative peptides for each protein and combined them in 12 artificial proteins. After their expression in E. coli using stable isotope labeled medium, they are mixed with the sample to be analyzed followed by tryptic digestion, generating peptides from the endogenous proteins as well as the stable isotope labeled standard. Using a mass spectrometry strategy called multiple reaction monitoring, specialized on the quantification of known peptides in complex samples, it is possible to compare the signal intensity of the standard and the endogenous peptides and therefore to quantify the absolute amount of almost all currently known lysosomal proteins in a single measurement. Due to the high sensitivity and selectivity of this mass spectrometric approach, it is not necessary to pre-fractionate or enrich samples and low µg amounts of sample are sufficient for one analysis.

We succeeded so far in the expression and purification of all 12 QConCats as well as the mass spectrometry assay development for more than 75% of the resulting peptides. We performed initial absolute quantification of 48 lysosomal proteins in mouse embryonic fibroblasts and are currently extending this list for the remaining proteins. These data present the so-far largest dataset for the absolute quantification of lysosomal proteins. Once fully established, the method will allow to absolutely quantify the known lysosomal proteome and therefore to investigate the absolute abundance and stoichiometry of lysosomal proteins without the need for pre-fraction or enrichment from minimal sample amounts.

O-8

AAV9-based gene therapy restores enzymatic activity in a mouse model for aspartylglucosaminuria

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Aspartylglucosaminuria (AGU) is an autosomal recessively inherited lysosomal storage disease. It is caused by the absence of functional lysosomal enzyme aspartylglucosaminidase (AGA), resulting in the accumulation of AGA substrate, aspartylglucosamine (GlcNAc-Asn) in different body fluids and tissues. In humans, AGU is a progressive disorder characterized by intellectual disability, skeletal abnormalities, and early mortality. Currently, there is no cure for AGU. Previous adenovirus-mediated gene therapy was demonstrated to be effective in locally reducing lysosomal storage in the brain (intraparenchymal route) and fully correcting it in liver (IV route) of AGU mice. Over the past decade, adeno-associated virus (AAV) gene therapy has progressed rapidly and that AAV9 vectors have been shown to confer a dramatic therapeutic improvement to neurological disorders. Therefore, we hypothesized that AAV9-based gene therapy may impart a therapeutic benefit to AGU mice. A construct carrying the codon-optimized human *AGA* gene was packaged into AAV9 capsids as a self-complementary (sc) genome. The scAAV9/*AGA* vectors were injected by tail vein into adult AGU mice at $2x10^{11}$ vg/mouse. Serum AGA activity and urine GlcNAc-Asn excretion were measured at one week before and multiple time points post injection. Our results clearly show that IV injection of scAAV9/*AGA* vectors dramatically increases AGA activity 1 week post injection to a super physiological level (treated KO vs KO vs Het mice: 736.4±46.9 vs 0 vs 10.0±1.3 nmol/24hr/ml serum). Serum AGA activity in about half of treated mice decreases but is still significantly higher than in heterozygous mice and those levels are sustained in all treated mice up to 32 weeks post injection. Urine GlcNAc-Asn excretion decreases substantially in scAAV9/AGA vector treated groups at 4 (treated KO vs KO vs Het mice: 13±14 vs 1132±473 vs 0 mg GlcNAc-Asn/g creatinine) and 8 (treated KO vs KO vs Het mice: 14±14 vs 786±429 vs 0 mg GlcNAc-Asn/g creatinine) weeks post injection. These results suggest that IV administration of scAAV9/AGA vectors can almost completely clear peripheral GlcNAc-Asn accumulation in AGU mice. Similar results have been achieved in mice dosed by lumbar intrathecal (IT) injection. In one mouse treated IT at the dose of $1x10^{11}$ vg, AGA activity was restored at physiological levels in both the brain (Het mouse vs KO vs treated KO: 271.2 vs 102.9 vs 235.1 µmol/h/g of protein) and the liver (Het mouse vs KO vs treated KO: 68.6 vs 7.0 vs 70.4 µmol/h/g of protein). Behavioral tests, imaging, and histopathological staining are being used to determine if these benefits extend to the central nervous system. The results from the study suggest that gene therapy could be considered for possible future human translation.

O-9

The Batten disease protein CLN3 is a lysosomal ion channel that regulates lysosomal response to swelling

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Despite identification of the gene causing juvenile neuronal ceroid lipofuscinosis (Batten or CLN3 disease) in 1995, the precise function of the lysosomal CLN3 protein has largely remained elusive. In a collaboration with the Cotman lab we recently reported the presence of elevated lysosomal Ca^{2+} in a neuronal cerebellar cell line containing the common 1kb deletion in the *Cln3* gene[1]. We now present evidence that this elevation in lysosomal Ca^{2+}, present also in patient fibroblasts harbouring different mutations in *CLN3*, is a direct consequence of loss of CLN3 function and contributes directly to disease pathogenesis and neuronal loss. During our investigations of the potential cause of this alteration in lysosomal Ca^{2+} homeostasis we unearthed key residues in CLN3 that are conserved in ion channels. Utilising nanodisc purified CLN3 we have confirmed that this protein can act as a voltage gated K^+ channel in planar lipid membranes and that K^+ accumulates within CLN3 lysosomes disrupting lysosomal ion homeostasis. Furthermore, we have evidence from mammalian, yeast and slime mould models that CLN3 is required for normalising lysosomal ion balance following lysosomal swelling. Ultimately, changes in lysosomal ion homeostasis in CLN3 disease have knock-on detrimental effects on endolysosomal function, autophagic vacuole clearance and ultimately excitotoxicity of CLN3 disease neurons. These phenotypes can be ameliorated using certain Ca^{2+} antagonists that target lysosomal Ca^{2+} channels and alter lysosomal membrane potential providing a novel new therapeutic strategy for this disease.

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O-10

Inhibition of NAADP mediated lysosomal Ca^{2+} signalling induces Niemann-Pick type C phenotypes in cells and animal models.

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Niemann-Pick type C (NPC) disease is a rare neurodegenerative disorder caused by mutation in one of two lysosomal proteins – the lysosomal membrane protein NPC1 or the lysosomal lumen protein NPC2. Children with NPC fail to reach developmental milestones, and suffer from cerebellar ataxia and dementia. At the cellular level, NPC is characterised by storage of cholesterol, sphingomyellin and glycosphingolipids, and impaired endocytic trafficking. Lysosomes in NPC1 null cells contain less Ca^{2+} than wild-type cells and release less Ca^{2+} in response to the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP). We have previously shown that this reduction in lysosomal Ca^{2+} content occurs within 30min after inhibition of the NPC1 protein, before lysosomal lipid storage or impaired endocytic trafficking. We now show that disruption of lysosomal Ca^{2+} signalling is central to NPC disease pathology and causes both lysosomal lipid accumulation and impaired endocytic trafficking.

NAADP is the most potent Ca^{2+} releasing second messenger in the cell. It causes the release of Ca^{2+} from the lysosome through two-pore channel 2 (TPC2). NAADP mediated lysosomal Ca^{2+} signalling can be inhibited using the selective NAADP receptor antagonist Ned19. Treatment with Ned19 leads to increased lysosomal volume, measured using lysotracker, intralysosomal accumulation of cholesterol, sphingomyelin and gangliosides, and impaired endocytic trafficking in RAW macrophages. Zebrafish embryos treated with Ned19 show reduced spontaneous Ca^{2+} oscillations and accumulate cholesterol, sphingomyelin and ganglioside in the brain. This combination of phenotypes is specific to NPC disease and confirms that altered lysosomal Ca^{2+} signalling is an early event in NPC pathogenesis which underlies subsequent lipid storage.

O-11

Mutations in ABCD4 disrupt interaction between the lysosomal proteins ABCD4 and LMBD1 involved in vitamin B_{12}-trafficking

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Vitamin B_{12} (cobalamin, Cbl), in the cofactor forms methyl-Cbl and adenosyl-Cbl, is required for the function of the essential enzymes methionine synthase and methylmalonyl-CoA mutase, respectively. Cbl enters mammalian cells by receptor-mediated endocytosis of protein-bound Cbl followed by lysosomal export of free Cbl to the cytosol, and further processing to these cofactor forms. The integral membrane proteins LMBD1 and ABCD4 are required for lysosomal release of Cbl, and mutations in the genes *LMBRD1* and *ABCD4* result in the cobalamin metabolism disorders cblF and cblJ. We report a new (fifth) patient with the cblJ disorder, who presented at 7 days of age with poor feeding, hypotonia, methylmalonic aciduria, and elevated plasma homocysteine and harbored the mutations c.1667_1668delAG [p.Glu556Glyfs*27] and c.1295G>A [p.Arg432Gln] in the *ABCD4* gene. Cbl cofactor forms are decreased in fibroblasts from this patient, but could be rescued by over-expression of either ABCD4 or, unexpectedly, LMBD1. Utilizing a sensitive live-cell FRET assay, we demonstrated selective interaction between ABCD4 and LMBD1, and decreased interaction when ABCD4 harbored the patient mutations p.Arg432Gln or p.Asn141Lys, or artificial mutations disrupting the ATPase domain. Finally, we showed that ABCD4 lysosomal targeting depends on co-expression of, and interaction with, LMBD1. These data broaden the patient and mutation spectrum of cblJ deficiency, establish a sensitive live-cell assay to detect the LMBD1-ABCD4 interaction, and confirm the importance of this interaction for proper intracellular targeting of ABCD4 and cobalamin cofactor synthesis.

O-12

Synthetic lethality between two lysosomal amino acid transporters

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The PQ-loop repeat-containing protein 2 (PQLC2; pronounced 'Picklock two') and cystinosin are lysosomal transporters which export cationic amino acids and cystine, respectively, from the lysosomal lumen for reuse in cell metabolism *(Jézégou et al. (2012) PNAS 109(50):E3434-43; Liu et al. (2012) Science 337(6092):351-4)*. Mice lacking cystinosin accumulate cystine in lysosomes and develop progressive dysfunction of diverse organs, but show normal survival rate *(Nevo et al. (2010) Nephrol Dial Transplant 25(4):1059-66)*. We established a constitutive knock-out (KO) model of PQLC2, which showed no obvious phenotype and a normal survival rate. However, intercross of the two mouse lines revealed a fully penetrant, embryonic synthetic lethality between the two lysosomal amino acid transporters. Double KO (DKO) embryos showed severe head and brain dysmorphia at embryonic days E10.5 and E12.5 of development. Of note, the DKO of cystinosin and the PQLC2 orthologue LAAT-1 is not lethal in *C. elegans* (*Liu et al. (2012) Science 337(6092):351-4*). The synthetic lethality observed in mice might thus be specific to vertebrates or mammals.

Our working hypothesis to explain this genetic interaction is that the activities of the two lysosomal transporters converge onto the synthesis of S-nitrosoglutathione and the modification of cellular thiols. According to this hypothesis, lysosomal export of arginine and cystine (reduced to cysteine) by PQLC2 and cystinosin, respectively, supplies cytosolic precursors of nitric oxide and glutathione to react with, and possibly protect, critical protein thiols. These S-nitrosylation and S-glutathionylation reactions would in turn either modulate metabolic/signaling pathways essential to mouse embryo development or protect embryo proteins from irreversible oxidative damage during the establishment of materno-fetal circulation.

O-13

Knock-in mouse reveals importance of slow gating of lysosomal H^+/Cl^- exchange in lysosomal function and bone resorption

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ClC-7 is a slowly voltage-gated H^+/Cl^- exchanger ubiquitously found in lysosomes and additionally at the ruffled border of bone-resorbing osteoclasts [1]. ClC-7 is required for normal degradation of endocytosed protein in lysosomes and for bone resorption. Hence, knockout mice of ClC-7 or its beta-subunit Osteopetrosis-associated transmembrane protein 1 (Ostm1) display severe osteopetrosis, a neuronal ceroid lipofuscinosis (NCL)-like lysosomal storage disease and die within the first six weeks of life [2-5]. 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 ClC-7, but some even increase the gating kinetics in a heterologous expression system [6]. We have recently found an accelerated mutant in osteopetrotic cattle that is expressed at normal levels and that is correctly localized to lysosomes in vivo [7], pointing towards a physiological importance of the slow gating. To investigate this role we have now generated a knock-in mouse model with the accelerating ClC-7 mutation S288F that is found in human osteopetrosis patients. Homozygous mice exhibit a severe osteopetrotic phenotype, no teeth eruption and a maximal lifespan of about four weeks. Furthermore, we found an alteration in lysosomal ion concentration. This mouse model will help to elucidate the role of the gating kinetics in the physiology of lysosomes and bone resorption, and to better understand the pathomechanism upon disturbances of lysosomal ion transport in general.

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O-14

Lysosomal malfunction impairs mitochondria via a transcriptional mechanism

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Lysosomal storage diseases are a group of pathologies whose primary cause is lysosomal dysfunction. Malfunctioning lysosomes have wide-ranging impacts in many aspects of cellular physiology, including other organelles. For example, mitochondrial impairments have been reported in many lysosomal storage diseases, including Pompe's, Niemann-Pick, Gaucher's and Batten's diseases among others. However, the molecular mechanisms underlying mitochondrial impairments in lysosomal storage diseases remain unclear, despite defects in mitophagy are often cited as the underlying cause. Here, we show that lysosomal malfunction triggers a transcriptional program that inhibits the biogenesis of new mitochondria. Furthermore, the removal of the transcription factors that coordinate this program rescues mitochondrial biogenesis and function.

We have employed mouse models of lysosomal malfunction (Gaa-/-, NPC1-/-) as well as patient fibroblasts to identify how mitochondria are impacted.

Using transcriptional data from NPC1-/- mouse tissues, we applied a multi-dimensional genomics strategy which revealed that the transcript levels of mitochondria-related genes decrease as the disease progresses. Using NPC1-/- MEFs as well as NP (Niemann-Pick) patient fibroblasts, and pharmacological models of NP, we determine that the progressive decrease in the expression of mitochondrial genes is due to the activation of transcriptional repressors, which we have identified. When we knock-down these repressors, the expression of mitochondria-related genes is returned to control levels, as well as mitochondrial function as measured by oxygen consumption. We further determined that these transcriptional repressors are activated in response to different types of lysosomal dysfunction. This defines a transcriptional program which, in response to lysosomal stress, triggers the repression of mitochondrial genes and a global down-regulation of mitochondrial metabolic roles. Finally, the rescue of mitochondrial function is also associated with a decrease in apoptosis, suggesting that mitochondrial malfunction contributes to the pathology of lysosomal storage diseases.

Our data mechanistically underpins how lysosomal dysfunction associated with lysosomal storage diseases results in the impairment of mitochondria.

This work was supported by a Starting Grant from the European Research Council (NR).

O-15

Generation of a Lysosomal Storage Disorder CRISPr Biobank for the study of Lysosomal Storage Disorders

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Lysosomal Storage Diseases (LSDs) are a group of rare, recessively inherited disorders, with an overall incidence of 1 in 5000. They are caused by mutations of genes encoding lysosomal proteins and are characterized by the progressive accumulation of un-degraded material in the lysosomes of many cell types and tissues. Although great advances have been done in the discovery of genes that are involved in LSDs, the mechanisms by which the storage of un-degraded material causes cellular and tissue dysfunction have yet to be fully elucidated. Furthermore, for most LSDs, any therapeutic options are inefficient or not available. A main limit in LSD research is the lack of robust and reliable cellular models suitable for both basic cell biology studies and/or for translational approaches such as high content drug screenings. For instance, skin-derived fibroblasts from patients and mouse models of LSDs, frequently used in LSD studies, often fail to show any significant phenotypes and display a high variability, which complicates any functional and translational analysis of such diseases. For this reason, we started the generation of novel cellular models for all known LSDs by engineering well-established, broadly used cell lines using the CRISPR-Cas9 technology. A preliminary phenotypic characterization revealed that most of the cell lines generated reproduce the disease phenotypes, including substrate accumulation, lysosomal enlargement and lysosomal dysfunction, which were rescued by reintroducing the WT gene, thus excluding the influence of any off-targets. Furthermore, we generated multiparametric high content experiments for the simultaneous analysis of lysosomal morphology, substrate accumulation, autophagy and other phenotypes , demonstrating that the cell lines generated are suitable for drugs screening experiments. Our main goal is the generation of a new LSDs CRISPr Biobank (LSD-CRIB) that will be used both for mechanistic studies and for drug screening approaches aimed at identifying novel compounds able to broadly induce intracellular clearance of the accumulated substrates. In addition, the collection of all validated KO cell lines for LSD genes will be catalogued in a database and made publicly available to the scientific community.

O-16

The lysosomal hydrolase Plbd2 - From knockout to functional role

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The lysosomal hydrolase Plbd2 (Phospholipase B domain containing 2) was identified in proteomic studies of M6P-containing proteins several years ago, and its lysosomal localization was confirmed later on[1]. Although the name suggests a function in phospholipid metabolism, we characterized Plbd2 as an Ntn-hydrolase by crystal structure analysis, suggesting a role in cleavage of non-linear amid bonds[2]. However, its physiological role could not yet be determined.

After synthesis and cleavage of the signal fragment in the ER, the 75 kDa Plbd2 precursor is targeted to the endo-lysosomal system, most likely due to a M6P-dependend sorting. In the acidic environment the precursor undergoes an autocatalytic cleavage, resulting in a 25 kDa α- and a β-fragment of about 40 kDa. The latter is further processed to generate a 15 kDa β1- and a 28 kDa β2-fragment. While the first cleavage occurs autocatalytically, the second cleavage step might depend on a cysteine-protease, as shown by its sensitivity to the cysteine-protease inhibitor E-64.

We failed to detect the cleavage of the β-fragment in AEP-deficient as well as in cathepsin (Cts) B/L-double deficient cells. As both cathepsins, but not AEP, are known to be sensitive to E-64 and furthermore, maturation of CtsB and CtsL was shown to be dependent on AEP-activity[3], we suggest a CtsB and/or CtsL-mediated maturation of Plbd2 rather than a direct AEP-mediated cleavage.

To investigate the physiological and pathophysiological relevance of Plbd2, we generated a Plbd2 knockout mouse model by CRISPR-Cas9 gene editing. To exclude functional complementation we also targeted the homologous lysosomal protein Plbd1 (35 % identity, 51% homology). Both single-knockout mouse models as well as the double-deficient knockout mouse model might help to elucidate the physiological substrate(s) and functional role(s) of these new lysosomal hydrolases.

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O-17

Arylsulfatase K (ARSK) – The missing link in glycosaminoglycan degradation. Arsk-knockout mouse characterization.

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The degradation of glycosaminoglycans (GAGs) involves a series of exolytic glycosidases and sulfatases that act sequentially on the non-reducing end of the polysaccharide chain. Enzymes have been cloned that catalyze cleavage of all known linkages with the exception of desulfation of 2-sulfoglucuronate, which is found in heparan sulfate and dermatan sulfate[1]. Using synthetic saccharides as substrates, we recently could show *in vitro* that the novel lysosomal arylsulfatase K (ARSK) acts selectively on 2-sulfoglucuronate and lacks activity against 2-sulfoiduronate[2]. As ARSK has all of the properties expected of a lysosomal enzyme[3], we conclude that Arsk is the long sought lysosomal glucuronate-2-sulfatase.

Sulfatases specifically hydrolyze sulfate esters in glycosaminoglycans, sulfolipids, or steroid sulfates, thereby playing key roles in cellular degradation, cell signaling, and hormone regulation. The loss of sulfatase activity has been linked to severe pathophysiological conditions such as lysosomal storage disorders (LSD), developmental abnormalities, or cancer[1]. Here we show initial results obtained from the analysis of an Arsk-knockout mouse. No significant evidence for central nervous system impairment and also no other obvious phenotype is being observed. qPCR analysis show elevated transcription levels of other lysosomal genes (i.e: *Hexb*, *Lamp1*). Increased amounts of excreted GAGs in urine and higher overall GAG levels in tissues (liver, kidney) are indicators for a LSD. Analysis of GAGs from isolated liver tritosomes indicates significant storage of heparan sulfate. EM analysis show dense bodies in kidney and chondrocytes as well as storage material filled vacuoles. Bone analysis reveals reduced bone to tissue (BV/TV) quotient that may lead to osteopenia. All these observations suggest a new form of a lysosomal storage disorder induced by the loss of arylsulfatase K function.

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O-18

Role of the gamma-subunit of GlcNAc-1-phosphotransferase in the pathogenesis of mucolipidosis type III

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Mucolipidosis III gamma (MLIII) is clinically characterized by onset of first symptoms at an average of 5 years such as stiffness of hands and shoulders, claw hand deformities, scoliosis and progressive destruction of hip joints. The disease is caused by mutations in *GNPTG* encoding the gamma-subunit of the GlcNAc-1-phosphotransferase complex. This enzyme is responsible for the generation of mannose 6-phosphate (M6P) targeting signals on 70 soluble lysosomal enzymes that are required for their efficient receptor-mediated transport to lysosomes.

Gnptg^{LacZ} reporter mice revealed expression of the gamma-subunit mainly in the central and peripheral nervous system, skeleton, lung, kidney and fat tissues. In fibroblasts from *Gnptg*^{KO} mice and in GNPTG-deficient human haploid HAP1 cells the GlcNAc-1-phosphotransferase activity is reduced leading to low amounts of M6P-containing proteins. M6P affinity chromatography-assisted and mass spectrometry-based secretome analysis of *Gnptg*^{KO} fibroblasts revealed a distinct set of lysosomal enzymes modified with M6P residues. Complementary SILAC-based lysosomal proteomics revealed decreased amounts of 28 lysosomal enzymes in *Gnptg*^{KO} fibroblasts involved in the degradation of lipids, glycans and proteins. Western blot analysis and measurements of lysosomal enzyme activities confirmed the missorting of specific lysosomal enzymes leading among others to the accumulation of lectin-positive storage material in lysosomes.

Gene array analysis of *ex-vivo* cultured bone-forming osteoblasts and bone-resorbing osteoclasts from wildtype mice revealed a cell type-specific expression of lysosomal enzymes. In addition to fibroblasts, the M6P formation and targeting of distinct lysosomal enzymes are impaired in primary *Gnptg*^{KO} osteoblasts and osteoclasts. Consequently, we found electron-lucent lysosomal storage material accumulating in bone and cartilage cells of *Gnptg*^{KO} mice that might impair bone remodeling. Moreover, *Gnptg*^{KO} osteoblast and growth plate chondrocytes showed elevated expression of the osteoclastogenic cytokine interleukin-6 (Il-6). The deep phenotypic analysis of the skeleton of *Gnptg*^{KO} is currently under investigation. Taken together, our data suggest a role of specific lysosomal lysosomal enzymes in bone and cartilage homeostasis, which might represent targets for therapeutic strategies in MLIII.

O-19

The fruit fly *Drosophila melanogaster* as a model system to study Gaucher disease

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Gaucher disease (GD) characterized by accumulation of glucosylceramide (GlcCer), mainly in cells of the reticuloendothelial system, is due to mutations in the *GBA1* gene. More than 800 mutations, mostly missense mutations, are known to date in the gene, leading to a heterogeneous disorder, that has been divided to a non-neuronopathic form, type 1 GD, and two neurological forms, which differ by severity, type 3 being less severe than type 2.

Aiming at studying the molecular and cellular abnormalities associated with GD pathologies, fly models have been thought recently[1-4]. The fruit fly has two *GBA1* orthologs*, GBA1a* (CG31148) and *GBA1b* (CG31414), each contains a minos element that leads to translation of a truncated GCase like protein. The *GBA1a* mutant protein has a C-terminal deletion of 34 amino acids, while the *GBA1b* mutant protein has a C-terminal, 129 amino acids deletion, which includes one amino acid of the active site. As a result, the GBA1a$^{m/m}$ flies are very mildly affected, while the GBA1b$^{m/m}$ flies present a severe neuronopathic disease.

In the present study, we show the spatial expression pattern of the two genes in normal and mutant flies and present data strongly arguing that both mutant proteins are expressed, with different activity toward an artificial substrate. Flies homozygous for mutant *GBA1a*^{m} GCase present some substrate accumulation, inflammation and activation of the unfolded protein response (UPR). Flies homozygous for the *GBA1b*^{m} GCase are severely affected, with substrate accumulation, inflammation and neuroinflammation and UPR activation.

Our results highlight the resemblance between the fly models and different GD forms and underlie their importance in future study of the disease as well as possible therapeutic modalities.

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O-20

The contribution of mutant glucocerebrosidase to the aggregation of alpha synuclein

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Gaucher disease (GD) is an autosomal recessive disease resulting from mutations in the acid β-glucocerebrosidase (GCase) encoding gene, *GBA1*, which leads to accumulation of the GCase substrate glucosylceramide mainly in monocyte derived cells.

GD patients and carriers of GD mutations have a significantly higher propensity to develop Parkinson disease (PD) in comparison to the non-GD population. This implies that mutant *GBA1* allele is a predisposing factor for 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 *GBA1* alleles, which leads to ER stress and UPR activation. Thus, flies expressing human mutant GCase variants in their dopaminergic cells exhibited death of dopaminergic cells, shorter life span and had a decreased negative geotaxis. ER stress and parkinsonian signs could be rescued by growing the model flies in the presence of the pharmacological chaperone ambroxol, which binds and removes mutant GCase from the ER.

One of the major characteristics of PD is the presence of insoluble oligomeric and fibrillar α-synuclein-positive inclusions known as Lewy bodies and Lewy neurites in neurons in the substantia nigra pars compacta. We could recapitulate the significant stabilization of α-synuclein and the existence of oligomeric and phosphorylated α-synuclein in the presence of mutant GCase in tissue culture dopaminergic cells expressing human mutant GCase variants.

In transgenic flies, expressing the human α-synuclein and human mutant GCase in dopaminergic cells, there was an earlier death of the cells and shorter life span in comparison to flies expressing only the mutant α-synuclein or in the presence of WT GCase.

Our results highlight the importance of mutant GCase in development of PD and strongly indicate its function in the toxic aggregation of α-synuclein.

O-21

Progressive lysosomal dysfunctioning inactivates muscle stem cells and blocks muscle regeneration in Pompe disease

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Skeletal muscle has the potential to efficiently regenerate after damage and this process is completely dependent on muscle satellite cells. It has been proposed but not proven that muscle wasting in muscle-degenerative conditions is the result of an imbalance between muscle damage and repair. Here we show that loss of regenerative potential tips the balance towards muscle wasting in a mouse model of Pompe disease (PD). PD is a recessively inherited metabolic myopathy caused by inactivating mutations in the acid alpha glucosidase (GAA) gene. GAA deficiency causes glycogen accumulation that is particularly damaging to skeletal muscle. Previously, we demonstrated that in muscle biopsies from Pompe disease (PD) patients satellite cells were present, but were not activated to repair the disease-mediated damage (Schaaf et al., 2015). In GAA-knockout (GAAKO) mice glycogen accumulation and lysosomal abnormalities are manifested directly after birth. During disease progression, we detected an increased number of Pax7 muscle stem cells, called satellite cells (SC). This expansion of the SC pool appeared to be caused by a continuous low-level SC activation, detected by the presence of Ki67-expressing SC, of a subset of GAAKO SCs during the first 5 months of life. At 5 months of age, Pax7+/Ki67+ SCs are lost, which coincides with the onset of decreasing fiber diameter and loss of muscle functioning that was determined in the Rotarod assay. The loss of GAAKO activation is a cell-extrinsic effect as GAAKO animals remain capable of repairing skeletal muscle after (repeated) chemical-induced injury, up to one year of age. Forced regeneration transiently reduced the lysosomal load and restored muscle functioning. We conclude that the satellite cell activation defect is due to an increased threshold for satellite cell activation as result of accumulated lysosomal damage. Identifying safe and efficient ways to re-activation satellite cells by exogenous signals may be used to improve the morphology and function of skeletal muscle from PD patients and positively affect their clinical course.

Schaaf GJ et al (2015) Acta Neuropathol Commun 3: 65

O-22

Mucopolysaccharidosis IIIA storage substrate drives an innate immune neuro-inflammatory response

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Mucopolysaccharidosis type IIIA (MPSIIIA) is a lysosomal storage disease characterised by mutations in the N-sulphoglucosamine sulphohydrolase (SGSH) gene, resulting in reduced lysosomal SGSH enzyme activity. Subsequently, an accumulation of highly sulphated, partially degraded glycosaminoglycans (GAGs) occurs in lysosomes and the extracellular matrix, alongside secondary accumulation of GM gangliosides, cholesterol and amyloid beta. MPSIIIA patients develop behavioural disturbances and progressive cognitive dysfunction, a possible consequence of neuro-inflammation. Brains from MPSIIIA mice demonstrate markedly increased astrocytosis and microgliosis, and coupled with up-regulation of IL-1β 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 GAGs and secondary storage substrates affect neuro-inflammatory pathways.

Here we demonstrate that highly sulphated GAGs from MPSIIIA mice specifically induced pro-inflammatory TNF-α and IL-1β responses when applied to a primary WT mixed glial culture. WT GAGs did not elicit a response. In addition, administration of MPSIIIA GAG *in vivo* stimulated analogous inflammatory responses. Both heparan sulphate (HS) and dermatan sulphate components of MPSIIIA GAGs, and 2-O sulphation of MPSIIIA HS is essential for pro-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 ($p \leq 0.001$). Monoclonal antibody blockade of CD14 and MD2, adaptor proteins of TLR4, significantly reduced the secretion of TNF-α. MPSIIIA GAGs were responsible for increased production of intracellular IL-1β, but alone failed to initiate IL-1β secretion. Secondary stimulation with MPSIIIA secondary storage substrates (ATP, cholesterol or amyloid beta), activated the NLRP3 inflammasome and initiated the release of intracellular IL-1β ($p \leq 0.001$). *In vitro* data suggests that MPSIIIA neuro-inflammation is 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, and whether modulation of the immune response will slow disease progression.

O-23

mTORC1 hyperactivation arrests bone growth in lysosomal storage disorders

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The mammalian target of rapamycin complex 1 (mTORC1) kinase promotes cell growth by activating biosynthetic pathways and suppressing catabolic ones, in particular (macro)autophagy. A prerequisite for mTORC1 activation is the translocation to the lysosomal surface. mTORC1 deregulation has been associated to the pathogenesis of several diseases, however its role in skeletal disorders is largely unknown. Here, we show that enhanced mTORC1 signaling arrests bone growth in lysosomal storage disorders (LSDs). We found that lysosomal dysfunction induces a constitutive lysosomal association and consequent activation of mTORC1 in chondrocytes, cells devoted to bone elongation. mTORC1 hyper-phosphorylates UVRAG, inhibiting phosphoinositide production by the Beclin 1-Vps34 complex leading to a block of the autophagy flux in LSD chondrocytes. As a consequence, LSD chondrocytes fail to secrete collagens, the main components of cartilage extracellular matrix. In mouse models of LSD, normalization of mTORC1 signaling or stimulation of the Beclin 1-Vps34-UVRAG complex rescued autophagy flux, restored collagen levels in cartilage and normalized bone growth. Taken together, these data unveil a novel role for mTORC1 in the pathogenesis of skeletal disorders and suggest new therapeutic approaches for the treatment of LSDs.

O-24

Multiplex LC-MS/MS lysosphingolipids analysis in plasma for the screening of sphingolipidoses and Niemann-Pick disease type C

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Lysosphingolipids have been recently reported as plasmatic biomarkers for various sphingolipidoses and Niemann-Pick disease (NP) type C.

A multiplex LC-MS/MS method for measuring lysoglobotriaosylceramide (LGb3), lysohexosylceramide (LHexCer); lysosphingomyelin (LSM), isoform 509 of LSM (LSM509), LysoGM1 and LysoGM2 gangliosides is in use since 2 years in our laboratory.

We report our results concerning patients affected with sphingolipidoses (untreated, n = 159), NPC (n = 55) compared to controls. The specificity was evaluated by testing samples from patients affected with other inherited disorders of metabolism.

- LHexCer is highly increased in Gaucher disease (GD, n = 27; mean 160 nmol/L, N <1.8) and saposin C deficiency (n = 1; 76 nmol/L), but can be very moderately increased in some cases. The lowest levels were found in patients homozygous for the mutation p.N370S.
- LHexCer is moderately increased in infantile Krabbe disease (n = 8; mean 13 nmol/L) but normal in 2 juvenile and 2 adult cases.
- LGb3 is elevated in Fabry disease (FD): highly in males affected with classical form (n = 15; mean 91 nmol/L, N<0.7), moderately in males with variant form (n = 10; mean 5.8 nmol/L) and in females heterozygous for classical form (n = 44; mean 5.7 nmol/L); it can be normal in females with variant form (4/8 cases; mean 1.4 nmol/L). Plasma LysoGb3 is a much more sensitive biomarker than urinary Gb3.
- LSM is increased in NP type A/B (n = 25; mean 16.5 nmol/L, N<1.1), sometimes very moderately (possibly normal, Kuchar et al, *Anal Biochem* 2017). In NPC (n = 55), LSM is normal in most cases (mean 0.9 nmol/L). LSM509 is highly increased in all NPA/B (mean 214 multiple of median of the controls of the day, MoM) and NPC <10 years (n = 26; mean 149 MoM) patients. In NPC > 10 years (n = 29), results are also clearly increased (mean 63 MoM), but sometimes moderately (at least 7 MoM) (see abstract M Pettazzoni).
- Abnormal presence of lysoGM1 and lysoGM2 is observed in most cases of GM1 gangliosidosis (5/6 cases), Sandhoff (7/9 cases) and Tay-Sachs diseases affected patients (3/4 cases), but not all (lack of sensitivity for mild and adult forms).

Measurement of sphingosine-1-phosphate (S1P), sphingosine and sphinganine can be added to this panel; slight modifications have been observed in plasma (*i.e.* slight increase of S1P in NPC).

Multiplex analysis of lysosphingolipids biomarkers in plasma is an efficient and rapid biochemical screening tool that can be used for diagnosis (particularly for the differential screening of NPC, NPA/B and GD) and for monitoring of patients receiving therapy (FD, GD). The same method can also be applied to various sample types.

O-25

Glycosphingoid bases (lyso-glycosphingolipids) in lysosomal storage disorders

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Prominent glycosphingolipidoses are Gaucher disease (GD; glucosylceramidosis), Fabry disease (FD; globotriaosylceramidosis) and Krabbe disease (KD; galactosylceramidosis) caused by deficiencies in glucocerebrosidase, α-galactosidase A and galacto-cerebrosidase, respectively. In these disorders, next to the accumulation of the primary storage glycosphingolipid, elevations in the corresponding glycosphingoid base (lyso-glycosphingolipids) are found to occur in plasma and urine. It has recently been recognized that active formation of sphingoid bases from accumulating storage lipids in lysosomes takes place by the action of acid ceramidase. The mass spectrometric measurement of glycosphingoid bases (glucosylsphingosine in GD; globo-triaosylsphingosine in FD; and galactosylsphingosine in KD) finds important applications in diagnosis, monitoring of disease progression and assessment of efficacy of therapeutic interventions. Accurate simultaneous quantitation of glycosphingoid bases in plasma and urine specimens has been developed employing ^{13}C-encoded identical internal standards. An overview is presented of the various procedures and applications. In addition, the potential use of isotope-encoded lipids in studies on *in vivo* metabolism of sphingolipids is demonstrated.

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O-26

Evaluation of Glucosylsphingosine as a Biomarker of the Eliglustat Treatment Response in Patients with Gaucher Disease Type 1 (GD1)

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Patients with Gaucher disease have deficient activity of acid β-glucosidase, resulting in accumulation of its major metabolite glucosylceramide (GL-1) and of glucosylsphingosine (lyso-GL-1), the deacylated form of GL-1, a minor substrate of the enzyme. Lyso-GL-1 is increasingly recognized as a reliable and highly specific biomarker of Gaucher disease that is associated with disease pathogenesis. We report on substantial decreases in lyso-GL-1 after 4 years of oral eliglustat treatment in two Sanofi-Genzyme-sponsored trials of treatment-naïve adults with Gaucher disease type 1 (GD1): an open-label Phase 2 trial (NCT00358150) in 26 patients, for which we report dried blood spot (DBS) values and a placebo-controlled Phase 3 trial (ENGAGE: NCT00891202) in 40 patients, for which we report plasma values. In both trials, decreases in lyso-GL-1 paralleled decreases in chitotriosidase and other Gaucher biomarkers and improvements in clinical parameters. In the Phase 2 trial, baseline median lyso-GL-1 levels in DBS (942 ng/mL, range: 248–2418) were more elevated than other biomarkers tested (165-fold compared to 47.2-fold for chitotriosidase, 13.4-fold for CCL18, and 1.8-fold for GL-1), with no overlap with normal values (5.7 ng/mL, range: 3.0–14.8). After 1 year of eliglustat treatment, the median DBS lyso-GL-1 value decreased by 61% (SD: 11.0%) and continued to decrease to a final median reduction of 83% (SD: 11.8%) at Year 4. Absolute decreases in DBS lyso-GL-1 correlated with absolute improvements in spleen volume (r=0.690), hemoglobin (r= 0.515), platelet count (r=-0.279), and chitotriosidase (r=0.386). Correlations between DBS lyso-GL-1 and liver volume and GL-1 were small and clinically insignificant. The association between lyso-GL-1 levels and clinical parameters (spleen and liver volumes, hemoglobin, platelet count) using a Repeated Measures Mixed Model were statistically significant ($P<0.05$) for all associations. In ENGAGE, the median baseline plasma lyso-GL-1 value of 350 ng/mL was elevated 70-fold compared to the normal range of <5 ng/ml. During the 9-month primary analysis, the median plasma value decreased by 48% in eliglustat-treated patients and was unchanged in placebo patients. After 4.5 years on eliglustat, the median plasma lyso-GL-1 value decreased by 84% relative to baseline. In summary, lyso-GL-1 levels decreased significantly in treatment-naïve GD1 patients after 4 or 4.5 years of treatment with eliglustat in both trials but did not fully normalize. Decreases in lyso-GL-1 levels correlated with improvements of the major clinical manifestations of the disease. Because it is in the causal pathway of GD1, lyso-GL-1 may prove to be a more useful marker of treatment response to eliglustat than chitotriosidase.

O-27

Urinary metabolomics and data modeling unveil mucopolysaccharidosis type I metabolic impairments

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Background: Application of metabolic phenotyping could expand the pathophysiological knowledge of mucopolysaccharidoses (MPS) and reveal the comprehensive metabolic impairments in MPS. However, few studies applied this approach to MPS.

Methods: We applied mass spectrometry-based targeted and untargeted metabolic profiling in urine samples obtained from a French cohort comprising 19 MPSI and 15 MPSI treated patients along with 66 controls. Furthermore, 24 amino acids have been quantified. Keratan sulfate, Heparan sulfate and Dermatan sulfate concentrations have also been measured. Univariate and multivariate data analyses have been used to select discriminant metabolites. The mummichog algorithm has been used for pathway analysis. Finally, a comparison of targeted and untargeted metabolomics data with in silico results has been conducted.

Results: The studied groups yielded distinct biochemical phenotypes using multivariate data analysis. Pathway analysis revealed that several amino acid pathways were dysregulated in MPSI.

Conclusion: This study constitutes one of the first metabolic phenotyping studies of MPSI. The findings might help to generate new hypotheses about MPS pathophysiology and to develop further targeted studies of a smaller number of potentially key metabolites.

O-28

Retrospective study of long-term outcomes of enzyme replacement therapy in Fabry disease: analysis of prognostic factors

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Despite enzyme replacement therapy, disease progression is observed in patients with Fabry disease. Identification of factors that predict disease progression is needed to refine guidelines on initiation and cessation of enzyme replacement therapy. To study the association of potential biochemical and clinical prognostic factors with the disease course (clinical events, progression of cardiac and renal disease) we retrospectively evaluated 293 treated patients from three international centers of excellence. As expected, age, sex and phenotype were important predictors of event rate. Clinical events before enzyme replacement therapy, cardiac mass and eGFR at baseline predicted an increased event rate. eGFR was the most important predictor: hazard ratios increased from 2 at eGFR <90 ml/min/1.73m^2 to 4 at eGFR <30, compared to patients with an eGFR >90. In addition, men with classical disease and a baseline eGFR <60 ml/min/1.73m^2 had a faster yearly decline (-2.0 ml/min/1.73m^2) than those with a baseline eGFR of >60. Proteinuria was a further independent risk factor for decline in eGFR. Increased cardiac mass at baseline was associated with the most robust decrease in cardiac mass during treatment, while presence of cardiac fibrosis predicted a stronger increase in cardiac mass (3.36 gram/m^2/year). Of other cardiovascular risk factors, hypertension significantly predicted the risk for clinical events. In conclusion, besides increasing age, male sex and classical phenotype, faster disease progression while on enzyme replacement therapy is predicted by renal function, proteinuria and to a lesser extent cardiac fibrosis and hypertension.

O-29 Lucerastat, an iminosugar for substrate reduction therapy: safety, tolerability, PD and PK in adult subjects with Fabry disease

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Background: Lucerastat, an inhibitor of glucosylceramide synthase (GCS), has the potential to provide substrate reduction therapy (SRT) in glycosphingolipid storage disorders such as Fabry disease (FD). The safety, tolerability, pharmacodynamics, and pharmacokinetics (PK) of oral lucerastat were evaluated in a Phase 1b study in adult FD subjects receiving enzyme replacement therapy (ERT).

Methods: In this single-center, open-label, randomized study, 10 subjects received 1000 mg lucerastat b.i.d. for 12 weeks on top of ERT (lucerastat group). Four subjects received ERT only (control group). The safety and tolerability of lucerastat, and effects on cardiac function, renal function, and biomarkers were evaluated every 4 weeks.

Results: Nine subjects reported 18 adverse events (AEs): 17 in the lucerastat group and 1 in the control group. A serious AE of atrial fibrillation was observed in the lucerastat group and part of medical history and, therefore, considered not related to lucerastat. No clinically relevant abnormalities in vital signs, safety laboratory, and 12-lead ECG were observed. Cardiac (left ventricular ejection fraction and myocardial mass index) and renal function (estimated glomerular filtration rate, urine albumin-to-creatinine ratio) remained stable. Plasma glucosylceramide (GlcCer), lactosylceramide (LacCer), and globotriaosylceramide (Gb3) were significantly decreased in the lucerastat group. At end-of-study, the mean (SD) change from baseline was -49.0 (16.5) % for GlcCer, -32.7 (13.0) % for LacCer, and -55.0 (10.4) % for Gb3 ($p < 0.0001$ for all). Urinary Gb3 was reduced by 52.5 (21.2) %. No statistically significant changes were observed for biomarkers in plasma and urine of the control group. PK parameters were comparable to those in healthy subjects.

Conclusions: 1000 mg b.i.d. lucerastat was well tolerated in FD subjects over 12 weeks. A marked decrease in plasma GlcCer, LacCer, Gb3, and urinary Gb3 was observed, indicating inhibition of GCS by lucerastat and suggesting clinical potential for SRT in FD.

***Support**: This study was funded by Actelion Pharmaceuticals Ltd, Allschwil, Switzerland. N. Guérard, O. Morand, R. Welford, and J. Dingemanse are currently employees of Idorsia Pharmaceuticals Ltd, the successor of Actelion Pharmaceuticals Ltd. C. Zwingelstein is a former of employee of Actelion Pharmaceuticals Ltd. C. Wanner received honoraria for consultancy from Actelion Pharmaceuticals Ltd.*

O-30

A BBB crossing peptide with lentiviral-mediated stem cell gene therapy fully corrects Mucopolysaccharidosis II mice

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Mucopolysaccharidosis type II (MPS II) is an X-linked lysosomal storage 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 in death by teenage years. Severe MPS II is non-responsive to enzyme replacement therapy or standard haematopoietic stem cell transplantation but remains the most frequent form of MPS II. Here, we report a novel lentiviral-mediated stem cell gene therapy approach to specifically target the brain using a blood-brain barrier (BBB)-targeting peptide. Haematopoietic stem cells (HSCs) were corrected using a lentiviral vector encoding the CD11b promoter and human IDS (LV.CD11b.IDS) or human IDS fused with a BBB peptide (LV.CD11b.IDS.Peptide), and transplanted into 6-8 week-old MPS II mice after full myelo-ablative conditioning using busulfan. LV.CD11b.IDS- and LV.CD11b.IDS.Peptide-transduced HSCs showed increases in IDS enzyme levels of 124-fold and 152-fold over wild-type, respectively, and complete engraftment into MPS II male mice at 8-weeks post-transplant. We achieved vector copy numbers in HSCs of 3.1 and 3.8 in LV.CD11b.IDS and LV.CD11b.IDS.Peptide groups, respectively. MPS II mice were evaluated for neurocognitive, skeletal and activity deficits using a series of behavioural tests and whole body x-rays at 8 months of age. We show complete correction of skeletal abnormalities and motor function using both lentiviral groups. Inflammatory cytokine profiles were normalised with LV.CD11b.IDS.Peptide in 8-months-old brains, and more importantly, cognitive deficits were fully corrected with LV.CD11b.IDS.Peptide but not LV.CD11b.IDS. These results provide excellent proof of principle for the translational outcomes of this approach for the treatment of the neurocognitive impairment in MPS II patients.

O-31

Combination of gene and enzyme replacement therapies for mucopolysaccharidosis type VI

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Enzyme replacement therapy (ERT) is the standard of care for several lysosomal storage diseases. ERT, however, requires multiple and costly administrations and has limited efficacy. We recently showed that a single high dose [2x10e12 genome copies (gc)/kg] administration of adeno-associated viral vector serotype 8 (AAV2/8) is at least as effective as weekly ERT in a mouse model of mucopolysaccharidosis type VI (MPS VI). However, the administration of high doses of AAV2/8 requires a challenging and costly production process and might result in both cell-mediated immune responses and insertional mutagenesis. We therefore evaluated whether the combination of low doses of AAV2/8 with a less frequent ERT schedule (monthly) than canonical (weekly) may be as effective as the single treatments at high doses or frequent regimen. We found that levels of correction in mice receiving the combined therapy were similar to normal controls as previously observed in mice administered with single treatments at high dose of AAV2/8 or weekly schedule of ERT. Since no amelioration in skeletal dysplasia was observed in adult mice treated with either high dose of AAV2/8 or frequent regiment of ERT, we are currently testing if combining weekly neonatal ERT and administration of 2x10e12 gc/kg of AAV2/8 in adult mice is more effective than each single treatment at improving bone abnormalities. Finally, we will investigate whether further reduction of ERT frequency is as much effective when combined with a single administration of AAV2/8. In summary, our data show that low dose gene therapy can be successfully used as a means to reduce the frequency of ERT administration. Based on this, further combinations of gene and enzyme replacement therapies deserve to be investigated to improve the efficacy of treatment for MPSVI and concomitantly reduce both the risks and costs associated with either therapy.

O-32

AAV9 gene transfer in Sandhoff mice : correction of brain and cerebellum using a combined way of administration

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Sandhoff disease (SD) is a genetic disorder due to mutations in the *HEXB* gene. It is characterized by a double Hex A (αβ) and B (ββ) 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. The murine model obtained by invalidation of the *Hexb* gene is a useful tool for the development of therapeutic approaches, as it exhibits a phenotype quite close to the human disease.

We decided to explore a gene transfer approach in SD based on the use of an AAV9 vector, known to cross the BBB after intravenous administration. A scAAV9-Hexb vector placed under the control of the phosphoglycerate kinase (PGK) promoter was intravenously administered in neonatal Hexb-/- mice. Animals treated with a dose of 3.5×10^{13} vg/kg had a survival similar to normal mice (>700 days) with no neurological sign by comparison with naïve Sandhoff mice. At 4 months post-treatment, lipid analyses using HPTLC showed that GM2 storage was absent in brain, but it is only decreased in cerebellum of treated mice. Even if no symptom was associated with this residual storage in mice at 2 years, we wondered if it could possibly be pathogenic at longer-term if extrapolated to patients. Therefore, we tested an intravenous (IV) + intracerebroventricular (ICV) combined way of administration using the same vector with the same final dose. Two groups of mice were injected using different doses in both compartments and treatment efficacy was evaluated at short- and long-term. Treated mice have now more than one year and they have no neurological sign. In both groups, hexosaminidases activities at short-term were around 30% of normal in treated mice brain. In cerebellum, a significant increase of enzymatic activity (around 20%) was obtained with the highest dose in the ICV compartment. In both groups, HPTLC analysis showed that GM2 storage was absent in normal as well as in AAV9-treated Sandhoff mice by comparison with naive mice. However, the highest ICV dose is needed to obtain a complete cure of the cerebellum. Neuroinflammation and autophagy dysfunction present in the cerebrum of Sandhoff mice were corrected in both groups after treatment. Short-term analyses were also performed on liver. Enzymatic activity was <10 % and GM2/GA2 gangliosides remained in treated mice. However, liver hypertrophy present in Sandhoff mice was significantly reduced in treated animals as well as lysosomal expansion. Our results showed that a minimal dose is required not only in ICV to obtain a prevention of GM2 accumulation in cerebellum but also in IV to completely correct liver.

O-33

Correction of neurological manifestations of MPSIIIC by a novel rationally designed neurotropic AAV gene therapy vector

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Mucopolysaccharidosis (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 results in widespread central nervous system pathology, behavioural problems, developmental delays, sleep disturbances and dementia. HGSNAT is a transmembrane protein; therefore secretion and cross-correction are unlikely, making the development of therapies challenging. Intracranial injection of AAV could potentially restore brain enzyme levels correcting neuropathology in MPSIIIC patients.

A novel AAV serotype (AAV-TT) was engineered to include key residues found in natural variants of AAV2, resulting in a gene therapy vector with extraordinary transduction characteristics in the CNS. We compared GFP expression of AAV9, Rh10 and AAV-TT in the brains of mice, demonstrating improved distribution of AAV-TT-GFP in the brain over AAV9-GFP and Rh10-GFP. AAV-TT-GFP specifically transduces neurons in the adult mouse brain.

We subsequently compared the therapeutic efficacy of AAV expressing the HGSNAT transgene using the two best serotypes AAV9-HGSNAT and AAV-TT-HGSNAT in a long-term study in MPSIIIC mice, delivered via bilateral intracranial injections. At 4 months post injection, behaviour was corrected in AAV-TT-HGSNAT treated MPSIIIC mice over AAV9-HGSNAT. At 6 months post injection, both serotypes restored HGSNAT enzyme activity levels in the brain, however, AAV-TT give significantly higher enzyme levels. Primary storage of total HS was decreased in the brains with AAV-TT-HGSNAT having no significant differences to WT. AAV-TT restored HS patterning over AAV9-HGSNAT. Secondary storage of GM gangliosides was corrected by both. Immunohistochemical analysis of the brain showed a greater reduction of inflammation in AAV-TT-HGSNAT treated mice. No anti-AAV IgG antibodies were detected against either serotype in all treated mice.

Our findings demonstrate that AAV-TT is better distributed within the brain and corrects the mouse model of MPSIIIC more effectively than AAV9. This is an improved vector design for diseases with global neuropathology such as MPSIIIC.

O-34 RNA based therapies for glycogenosis type II due to the common c.-32-13T>G mutation

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Glycogenosis type II (G2) is a lysosomal disorder caused by the deficient activity of acid alpha-glucosidase (GAA) enzyme, leading to the accumulation of glycogen within the lysosomes. Clinically, G2 is characterized by a highly variable phenotype ranging from a rapidly progressive infantile-onset (IO) to a slowly progressive late-onset form (LO). The c.-32-13T>G mutation, within intron 1 of GAA gene, is the most common mutation among LO-G2 patients. This variant affects the binding of the U2AF65 splicing factor to the polypyrimidine tract of exon 2, leading to a complete or partial exclusion of this exon from the mature *GAA* mRNA. However, in the presence of this mutation, variable levels of the normal spliced GAA transcript and protein are produced.

The aim of this study was to develop Antisense Morpholino Oligonucleotides (AMOs) to inhibit potential silencer elements present within the exon 2 of GAA and rescue normal splicing in the presence of the c.-32-13T>G mutation.

In order to identify potential silencer sequences within the exon 2 region, we first developed a fluorescence based splicing reporter system by creating a HeLa cell line stably transfected with a construct bearing the exon 2 of GAA and the flanking intronic regions (containing the mutation) cloned in the middle of the EGFP cDNA. Using this system we performed a highthroughput screening (HTS) of a library containing more than 50 siRNAs directed towards RNA binding proteins. Silencing of several splicing factors resulted in a significant increase of exon 2 inclusion, suggesting that these factors promote exon exclusion through their binding to silencer elements located within exon 2. Considering this result, we then developed a minigene system containing *GAA* exon 2 and the flanking intronic regions containing the mutation, bearing deletions of different regions of the exon. Using these minigenes, we identified 2 potential silencer sequences within this exon 2 (nt 147-286 and 357-500). Therefore, we designed a series of AMOs to specifically target both identified silencer sequences. An initial screening using a MUT minigene system led to the identification of a combination of 3 AMOs (1+2+3) directed towards the 147-286 exonic region as the best performing strategy to rescue exon 2 inclusion. By using this combination of AMOs, we successfully increased inclusion of exon 2 in the GAA mRNA and GAA enzyme activity both in patient's fibroblasts and myotubes. Most importantly, treatment of patient's myotubes resulted in a significant decreased of glycogen accumulation.

Taken together, these data suggest that the use of antisense oligonucleotides technology represent a promising strategy for the treatment of *GAA* patients carrying the common c.-32-13T>G mutation.

O-35

Neonatal umbilical cord blood transplantation halts disease progression in the murine model of MPS-I

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Umbilical cord blood (UCB) is a promising source of stem cells to use in early hematopoietic stem cell transplantation (HSCT) approaches for several genetic diseases that can be diagnosed at birth. Mucopolysaccharidosis type I (MPS-I) is a progressive multi-system disorder caused by the deficiency of the lysosomal enzyme α-L-iduronidase, and patients treated with allogeneic HSCT at the initial stages of the disease have improved outcome, building a strong rationale to administer such therapy as early as possible. Given that the most representative MPS-I murine models are immunocompetent mice, we here developed a transplantation model based on murine UCB. With the final aim of testing the therapeutic efficacy of UCB in MPS-I mice transplanted at birth, we first characterized murine UCB cells and demonstrated that they are capable of multi-lineage hematopoietic repopulation of myeloablated adult mice to an extent comparable with bone marrow cells. Donor-derived cells isolated from primary recipients were able to radioprotect and reconstitute multi-lineage hematopoiesis in secondary recipients. We then assessed the effectiveness of murine UCB cells transplantation in busulfan-conditioned newborn MPS-I mice. Twenty weeks after treatment, iduronidase activity was increased in the organs of MPS-I animals, and glycosaminoglycans storage was reduced. Affected mice with engraftment levels ≥50% showed improvements in the skeletal phenotype compared to untreated controls, as attested by radiographs, micro-computed tomography, and histopathology. This study explores a potential therapy for MPS-I at a very early stage in life and represents a novel model to test UCB-based transplantation approaches for various diseases.

O-36

Non-depleting anti-CD4 monoclonal antibody induces immune tolerance to enzyme replacement therapy in a Mucopolysaccharidosis type I mouse model

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Mucopolysaccharidosis type I (MPS I) is an autosomal recessive lysosomal storage disease (LSD) caused by the deficiency of α-L-iduronidase (IDUA), resulting in the lysosomal accumulation of heparan sulphate and dermatan sulphate [1]. Currently, the two therapeutic strategies available for MPS I patients are haematopoietic stem cell transplant (HSCT) and enzyme replacement therapy (ERT). Over 80% of MPS I patients develop high antibody titres to ERT, with 35% of these incidences being significantly inhibitory. High inhibitory antibody level was found to correlate with poorer treatment outcomes, and HSCT is the only reported effective immune tolerance regimen for MPS I patients currently. [2,3,4].

In order to develop an effective immune tolerance regimen for MPS I patients, immune tolerance induction approaches that were reported to increase T regulatory and B regulatory cell production, were evaluated in a MPS I mouse model in this study: 1) combination of non-depleting anti-CD4 and anti-CD8 monoclonal antibodies (mAb), 2) methotrexate, 3) methotrexate with anti-CD4 and anti-CD8 mAbs, 4) anti-CD4 mAb and 5) anti-CD8 mAb. All treated mice received 0.58 mg/kg of Aldurazyme at weekly intervals for 10 weeks, and Aldurazyme was delivered via a Sigma Adjuvant System on day 49 in order to further challenge the immune system. Most immune tolerance regimens tested were able to reduce antibody responses to Aldurazyme, even though some were more effective than others. In particular, two courses of anti-CD4 mAb were able to ablate immune responses to Aldurazyme in seven out of eight MPS I mice (85%). This suggests that anti-CD4 mAb can be an effective alternative immune tolerance regimen to ERT.

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O-37

Immune Tolerance Induction by Lentiviral Stem Cell Gene Therapy in Pompe Disease

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Pompe disease is characterized by generalized glycogen accumulation due to deficiency of acid α-glucosidase (GAA) in the lysosome. It is life threatening in classic infantile patients due to cardiorespiratory insufficiency caused by progressive pathology in cardiac and skeletal muscle. The current standard of care is enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA). However, one of the major complications of ERT is antibody formation that can severely impair its therapeutic efficacy especially in classical infantile patients. Hematopoietic stem cell mediated lentiviral gene therapy provides an attractive alternative treatment for Pompe patients, not only because of its therapeutic value but also due to its beneficial role in inducing immune tolerance. In this study, we focused on immune modulation and demonstrated that full prevention of antibody formation against both the transgene product and rhGAA was established within 4 weeks in a Pompe mouse model. The induced immune tolerance was complete at 4 weeks after gene therapy, which at subtherapeutic conditions was sufficient to prevent antibody formation to regular ERT dosing and five-fold higher dosing of ERT. Importantly, by generating immune tolerance, subtherapeutic lentiviral gene therapy allowed ERT to be effective with full phenotypic correction in cardiac and skeletal muscle. Our results highlight the possibility of clinical application of lentiviral gene therapy for Pompe disease, which in case of a suboptimal gene therapy outcome, induces immune tolerance to prevent interference of antibodies with ERT.

O-38

AAV gene transfer halts disease progression in clinically affected sheep with CLN5 Batten disease

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The lysosomal storage diseases, the neuronal ceroid lipofuscinoses (NCLs; Batten disease) are the most common degenerative brain diseases in childhood. At least 13 different causal genes have been implicated (designated *CLN1-8*, *10-14*). Despite this genetic diversity, the NCLs are defined by similar pathological and clinical features, namely the near-ubiquitous accumulation of lysosome-derived storage bodies, progressive neuronal loss and retinal degeneration, seizures and psychomotor decline culminating in premature death. Currently there are no effective treatments however encouraging translational studies in animal models, including sheep, have resulted in several gene therapy and enzyme replacement clinical trials now underway.

Sheep with naturally occurring CLN5 disease are ideal candidates for testing gene therapies. Their gyrencephalic brains are similar in physical organisation to human brains and in size to non-human primates thus they provide good surrogates for estimating the dose requirements and vector distribution for humans. Additionally CLN5 affected (*CLN5*$^{-/-}$) sheep share the main neuropathological features of the human disease. We have previously shown that the single intracranial administration of an AAV9 vector encoding ovine *CLN5* into 3 month old pre-symptomatic *CLN5*$^{-/-}$ sheep provided protection against stereotypical disease, the only clinical sign being a much delayed-onset loss of vision. Whilst therapeutic intervention at the earliest possible time is desirable, the diagnosis of NCL in humans normally follows clinical presentation and can be prolonged. Here, in a more clinically relevant setting, 7 month old *CLN5*$^{-/-}$ sheep with established disease symptoms and overt neurodegenerative changes received similar corrective gene therapy. Monthly clinical assessments and maze tests showed that the treatment halted any further decline in motor, neurological or behavioural capability over the next 14 months. Although treated sheep lost their vision, they remain healthy in the field at 21 months of age. In contrast, untreated *CLN5*$^{-/-}$ sheep developed advanced disease symptoms, with manifest seizure activity, and did not survive beyond 21 months. Longitudinal computed tomography (CT) scans indicated little further post-injection brain atrophy in the treated sheep.

Monitoring of these sheep continues, to determine if there are any signs of late-onset disease and to see if another dose of gene therapy may be warranted. Together, these data in both pre- and post-symptomatic sheep provide a strong rationale for clinical translation to CLN5 affected human patients.

O-39

BATCure: An H2020 Consortium developing new therapies for Batten disease

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The neuronal ceroid lipofuscinoses (NCL, Batten disease) are a group of monogenic inherited neurodegenerative lysosomal diseases. Thirteen genes have been identified and >400 mutations that cause an ever-widening spectrum of phenotypes, including cases of adult onset visual failure only. BATCure is a comprehensive programme that includes identifying new target pathways, extending the natural history, and developing drug and gene treatments for patients living with three genetic types that affect more than 50% of all children and young adults with NCL. They include the most prevalent type of NCL, juvenile CLN3 disease, and about half of all adult-onset cases.

As a step towards developing therapies for the brain, and necessary as the eye is a protected environment, we use Adeno-associated virus (AAV)-mediated gene therapies to restore protein expression in the NCL retina. We began with the *Cln6*^{nclf} mouse that has rapid loss of photoreceptor cells and photoreceptor function. Curiously, the expression level of *CLN6* is low in photoreceptors and high in bipolar cells, a cell type of the inner retina that receives input from photoreceptors and transmit signals to retinal ganglion cells. We performed subretinal injections of AAV8.CLN6 in pre-symptomatic mutant animals to treat the photoreceptors, as is successful in other genetic loss of vision diseases. Despite a widespread expression of *CLN6* in the treated eyes, no therapeutic effect was observed indicating that supplementation of *CLN6* in photoreceptors is not sufficient to prevent the retinal degeneration. Since conventional AAV serotypes poorly transduce bipolar cells, we injected *Cln6*^{nclf} mice with 7m8 carrying *CLN6* and a ubiquitous promoter to drive expression in cells of all retinal layers, including bipolar cells. Treated mice had a significantly thicker photoreceptor layer and enhanced photoreceptor function up to 9 months post viral vector administration compared with untreated mutants. To assess further which cell type of the retina is essential for the therapeutic effect, we administered 7m8.CLN6 vector carrying the bipolar cell type-specific promoter Pcp2 or Grm6. This specific expression of *CLN6* in bipolar cells led to increased photoreceptor function and thickness of the photoreceptor layer comparable with eyes treated with 7m8.CMV.CLN6. These data establish that the transduction of bipolar cells is essential to correct the photoreceptor degeneration in *Cln6*-deficient mice. This is the first study demonstrating that the deficiency of a gene highly expressed in bipolar cells can cause photoreceptors to die through a so far unknown mechanism.

Similar work in the eye is ongoing for four NCL genes, and in the brain and whole body for five NCL genes.

Abstracts of Poster Presentations

(by alphabetical order of first author)

P-1

Simple fluorimetric test for lysosomal swelling as a means to identify, monitor and develop therapies for all lysosomal storage diseases

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Lysosomal storage disorders (LSDs) are a group of ~70 severe neurodegenerative diseases that are a common cause of early death in children. The prevalence of LSDs is approximately 1 in 5,000 live births [1] . Therapies for LSDs have been developed in the areas of enzyme replacement therapy, hematopoietic stem cell transplantation and substrate reduction therapy [2]. However, up until now the screening and monitoring strategies for lysosomal diseases have relied heavily on symptom identification, coupled with enzymatic assays, followed by genetic screening of known mutations. This process can be lengthy, which in some cases, e.g. Niemann-Pick C disease, can affect therapeutic efficacy [3]. Therefore, the high prevalence of LSDs alongside continuing development of therapies requires the development of reliable biomarkers that can be used to detect LSDs in high risk populations, or that can be used for newborn screening.

LSDs are caused by defects of lysosomal function, commonly mutations in genes that encode catabolic enzymes involved in the degradation of macromolecules [4]. The storage of these substrates causes an expansion in late endosomes and lysosomes , in terms of size and number. Lysotracker has been developed as a method to monitor patients with Niemann-Pick C disease [4,5]. We have further developed this assay by identifying the mechanisms by which lysotracker loads into lysosomes . That was achieved by investigating in depth the factors that may affect these probes' efficacy, e.g. concentration, stability, pH, fixation,etc. To utilise lysotracker as a simple and rapid assay, we have adapted the method from microscopy and flow cytometry to a higher throughput 96-well plate technique that would be suitable for high throughput drug screening or for use in basic clinical laboratories.

We have further adapted the assay for 96-well plate based magnetic capture of circulating *$CD19^+$ B cells* from whole blood. These findings suggest this assay could be used for monitoring disease progression, response to therapies and as a drug screening tool.

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P-2

The Broad Clinical Variability of GM1 Gangliosidosis

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Purpose: Deficiency of the lysosomal enzyme β-galactosidase may cause three different diseases: GM1 gangliosidosis, mucopolysaccharidosis type IV B, or galactosialidosis. The deficiency of ß-galactosidase leads to an accumulation of GM1-gangliosides and/or keratansulfate. Patients with GM1 gangliosidosis develop progressive neurodegeneration and skeletal abnormalities. Depending on the onset of the disease and the clinical picture, GM1 gangliosidosis may be classified into infantile, late-infantile, juvenile, and adult onset forms. Purpose of this study was the evaluation of the variability of clinical symptoms in patients with different forms of GM1 gangliosidosis.

Methods and patients: We retrospectively analyzed the clinical data of 22 patients with confirmed diagnosis of GM1 gangliosidosis from 5 different metabolic centers in Germany and Austria. Data analyses included medical history, clinical and diagnostic parameters.

Results: 7 patients were classified as infantile, 12 patients as late-infantile, and 3 patients as juvenile form of GM1 gangliosidosis. Patients with the infantile form presented with muscular hypotonia as the first symptom at the age of 2.0±1.97 months. Patients with the late-infantile form presented with variable motoric and non-motoric neurologic symptoms, i.e. cognitive deterioration, spasticity, pyramidal signs, dystonia, and dysarthria at the age of 19±8.2 months. Patients with the juvenile form developed gait abnormalities at the age of 36±6.9 months.

All patients with infantile GM1 gangliosidosis presented coarse facial features, and only 3 out of them revealed cherry-red spots. Patients with the late-infantile or juvenile form showed neither coarse facial features nor cherry-red spots. Patients with the juvenile form did not present any visceral symptoms. However, a cognitive decline was noted in all patients. Epileptic seizures and dysostosis multiplex were diagnosed in all forms of GM1 gangliosidosis (8/22 patients, and 8/13 patients, respectively). The time between first symptoms and diagnosis was 6±2.4 months in patients with the infantile form, 2.4±4.3 years in patients with the late-infantile form, and 14±4.3 years in patients with the juvenile form.

Conclusion: The time of diagnosis in patients with disease onset of GM1 gangliosidosis beyond the first year of life is markedly delayed. Most likely, the broad variability of clinical symptoms may cause this delay. GM1 gangliosidosis should be considered in all patients presenting with progressive neurodegeneration and spastic-dystonic movement disorders of unknown origin, even in the absence of visceral symptoms or

cherry red spots. Early diagnosis is important as new therapeutic options are in development.

P-3

Characterising the fundamental cell biology of CLN8 disease for the purpose of drug screening and development

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The neuronal ceroid lipofuscinoses (NCLs) are a group of childhood-onset neurodegenerative lysosomal storage diseases (LSDs) characterised by the accumulation of autofluorescent lipopigment in a variety of cells and tissue particularly neurons. Mutations in 14 different causative genes (CLN1-CLN14) have been identified. Our research focuses on CLN8 disease, generally referred to as epilepsy with progressive mental retardation (EPMR) and variant late infantile NCL, both of which are caused by mutations in the CLN8 protein. Whilst the function of CLN8 is unknown, it has been identified as a transmembrane protein located in the endoplasmic reticulum (ER). It is recycled between the ER and ER-Golgi intermediate compartment. The pathogenic mechanisms in both EPMR and variant late infantile NCL are still unclear. To find therapeutic targets in the disease and across the LSDs, the pathophysiological mechanisms of these diseases must first be understood. Our project focuses on uncovering and characterising the cellular phenotypes in CLN8 disease by measuring organelle localisation, organelle structure, organelle accumulation, alteration in cellular lipid levels, endocytosis and Ca^{2+} signalling. Our data has revealed that, unlike most of the LSDs, CLN8 disease patient cells do not seem to exhibit lysosomal expansion and accumulation. They do, however, show an increase in ER density, mitochondrial numbers and EEA1 staining, which suggests that ER, mitochondrial and early endosome accumulation and/or expansion. Interestingly, our research has found that the severity of these phenotypes changes over a 24-hour period, which could indicate a circadian rhythm effect on the expression of mutant CLN8 protein. Currently, we are further investigating cellular phenotypes in CLN8 disease patient cells, such as differences in lipid metabolism and endocytosis. we aim to identify the earliest pathogenic event in the disease cascade and find common phenotypes across the LSDs to identify potential therapeutic interventions.

P-4

EXTL2 as a target for substrate reduction therapy in iPSC-derived neurons from Sanfilippo C patients

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Sanfilippo syndrome type C is a rare lysosomal storage disorder caused by mutations in the *HGSNAT* gene, which encodes an enzyme involved in heparan sulphate (HS) degradation. Mutations in this gene cause the storage of this substrate inside the lysosome. The disease has an autosomal recessive inheritance pattern and is characterized by a severe and progressive neurodegeneration for which no effective treatment exists.

Previously, we demonstrated, on Sanfilippo C patients' fibroblasts, that the use of siRNAs targeting *EXTL2* and *EXTL3*, genes involved in HS synthesis, could be effective as a short-term substrate reduction therapy (SRT) [(1)].

Here, we have used five different lentiviral vectors encoding shRNAs targeting *EXTL2*, to analyse the effect of this SRT as a long-term treatment for this and other Sanfilippo subtypes. First, we performed the assays on patients' fibroblasts. We could observe that all the shRNAs caused a notable reduction in the mRNA levels (around 90%) of the *EXTL2* gene sixty days post-infection. Moreover, immunocytochemistry analyses showed a clear decrease of the HS amounts after treatment.

Due to the good results obtained, now we are using the most effective shRNAs (sh4 and sh5) on neurons derived from patients' induced pluripotent stem cells (iPSC) [(2)]. We are using an established protocol for the differentiation of those iPSC into neurons [(3)], the most affected cell type in this disease. This protocol is based on the overexpression of an important transcription factor involved in neurogenesis. With this protocol, neurons are obtained within a week and they show functionality and maturation after one month. We consider that the use of this technique will greatly accelerate the assessment of any treatment.

To evaluate this therapeutic option, we are performing the same assays that we carried out in fibroblasts, but in the iPSC lines (infected with shRNAs or not) differentiated into neurons. Thus, we are analyzing different aspects of the effectiveness of the shRNAs in neurons, such as the percentage of inhibition of the *EXTL2* gene at mRNA level (using qPCR), the accumulation of HS over time (with inmunocytochemistry) or the levels of neuronal activity in neurons (using calcium imaging).

Our preliminary results in patients' fibroblasts confirm the usefulness of shRNAs as a long-term SRT, and make their use a promising approach for a future therapeutic option for Sanfilippo C syndrome.

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P-5

Activity of lysosomal glucocerebrosidase towards xylosides

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Glucocerebrosidase (GBA) is a lysosomal β-glucosidase that is deficient in Gaucher disease (GD; glucosylceramidosis). The clinical manifestations associated with GBA deficiency in GD patients are remarkably heterogeneous, involving various tissues with variable severity. Correlation of GBA genotype and GD manifestations is not strict [1]. Moreover, carriers of a mutant GBA allele are at increased risk for Parkinson's disease [2].

The present lack of understanding of the clinical outcome of abnormalities in GBA prompted us to re-investigate catalytic features of the enzyme. Recently we identified the formation of glucosyl-cholesterol by GBA as the result of a transglycosylation reaction [3]. Attention was next focused to glycon specificity of GBA. Here, it is demonstrated that GBA is able to hydrolyze 4MU-β-xyloside, although with lower affinity than 4MU-β-glucoside. Moreover, it is shown that GBA can perform a transxylosylation reaction, being able to generate xylosyl-cholesterol in vitro as well as intact cells. Sensitive mass spectrometric detection of these novel metabolites was developed. The physiological relevance of metabolism of xylosides by (mutant) GBA is topic of ongoing research.

In conclusion, examination of metabolism of xylosides by GBA warrants further examination.

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P-6

A cellular model of Neuronal Ceroid-Lipofuscinosis type 3 created by CRISPR-Cas9 provides new insights into disease pathogenesis

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Neuronal Ceroid-Lipofuscinosis type 3 (NLC-3), also known as Batten Disease (BD), is a severe neurodegenerative Lysosomal Storage Disease (LSD) caused by mutations in the *CLN3* gene, with an early onset that, in children, rapidly induces visual loss, seizures, mental deterioration and death. A typical clinical manifestation of the disease is the intra-neuronal accumulation of autofluorescent granules made up of proteins and alchool-insoluble lipids (known as lipofuscins). Up to date, the role of CLN3 protein has been highly debated, and this made any attempt for the generation of novel therapeutic approaches extremely difficult. We recently generated, taking advantage of the CRISPr/Cas9 technology, a new cellular model of NCL-3 to investigate the disease phenotypes. These cells are *CLN3*-KO and show increased LAMP1 levels and lysosomal aggregation, clusterization and impaired degradation abilities. The lysosomal associated disease-phenotype could be rescued by a *CLN3*-3XHA construct, leading to lysosomal dispersion and reduced LAMP1 protein levels. We demonstrated that our cell line is a good disease model, as it recapitulates the major disease-phenotype, thus accumulating lipofuscins after blocking the cell cycle by Hydroxyurea treatment. Study of the autophagy flux revealed that the chronic removal of the *CLN3* gene does not affect autophagosomes biogenesis or fusion with lysosomes, but it does affect the degradation ability of these autophagosomes and autolysosomes, that consequently accumulate inside the cell. Once established that both lysosomal and autophagic machineries are engulfed, we also assessed the endocytosis function by looking at chlatrin-mediated endocytosis, through EGF stimulations.

These treatments revealed that endocytosis of the EGF molecule is not altered in *CLN3*-KO cells, but AKT phosphorylation is highly impaired, suggesting a defect in the PI3K/AKT axis.

We also observed an impairment of the cell proliferation rate in the *CLN3*-KO population, that can be a consequence of the reduced AKT phosphorylation.

In conclusion, the generation and study of this molecular tool will help us to unravel the *CLN3* gene function, and to understand how this can be linked to the physio-pathological events associated with the disease development and progression.

P-7

Circulating glycosphingolipids in patients with GM2-gangliosidosis

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The GM2 gangliosidoses, which include Tay-Sachs disease, Sandhoff disease and the AB variant, are rare lysosomal storage disorders causing progressive neurodegeneration, and in Sandhoff disease, organomegaly. They are caused by a functional deficiency in the lysosomal enzyme β-hexosaminidase, which results in an inability to breakdown glycosphingolipids (GSLs) with terminal hexosamine sugars, typically causing progressive neurodegenerative disease. We used a highly sensitive and robust HPLC method developed in our lab, to quantify GM2 and other GSLs, in plasma samples from 29 patients with GM2 gangliosidosis. We evaluated the relationship between GSL storage and various clinical measurements to determine the potential usefulness of plasma GSL quantification to monitor disease progression and response to treatment in patients. We also measured GSLs in samples taken over the course of a clinical trial with pyrimethamine in a late-onset Tay Sachs patient. We found that plasma concentrations of GM2, GA2, Lc3 and Gb4 were significantly elevated in patients compared to controls, and that GA2, Lc3 and Gb4 were significantly elevated in Sandhoff patients compared to Tay-Sachs. There was no clear relationship between circulating concentrations of most GSLs and either age at diagnosis, or residual β-hexosaminidase activity.

P-8

Identification of disorders of glycoprotein degradation and other related diseases using a new HPLC method.

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Disorders of glycoprotein degradation are a group of lysosomal diseases caused by an enzymatic deficiency of the catabolism of the oligosaccharide part of N-glycoproteins. In other related diseases, such as gangliosidosis GM1 type 1, the enzyme activity not only affects glycoproteins, but also gangliosides. As a consequence of the enzymatic failure, in all of these lysosomal defects, there is a urinary increase of certain oligosaccharides.

Historically, the most widespread biochemical screening method of these pathologies has been thin layer chromatography on silica gel plates. However, during the last years, faster, less laborious and more automated methodologies have been appearing in order to be able to screen a large number of patients. This need arises from the fact that clinical diagnosis is very difficult, due to the great variability of symptoms in patients with these diseases and the need to be able to diagnose and intervene as early as possible.

In the present work we describe a separation method based on HPLC for the identification of different oligoscaridoses. The results show specific profiles of oligosaccharides in the different N-glycoproteins analyzed, as well as in other diseases such as gangliosidosis GM1 type 1. In parallel, this method allows the quantification of free sialic acid, an increased biomarker in lysosomal diseases due to the accumulation of this acid (the infantile sialic acid storage disease and the Salla disease).

P-9

Effects of high-oral ambroxol chaperone therapy in two Italian patients with type 3 Gaucher disease

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Ambroxol in combination with enzyme replacement therapy (ERT) has proven to be a promising therapy for patients with neuronopathic Gaucher disease (GD3) (Narita et al). We started ambroxol therapy coupled to ERT in 2 patients with GD3.

The first patient is a girl diagnosed with Gaucher disease when she was 6. Her genotype was N188S/IVS2+1G>A, a neurologic mutation. ERT was started immediately after diagnosis. Neurological examination was normal, as well as neurological investigations (EEG, IQ, brain MRI). During the follow up, her EEG became increasingly pathologic; at 14 she started to have seizures. Ocular abnormalities, i.e. pathological saccadic movements, were highlighted, IQ remained normal. She started to be treated with sodium valproate, levatiracetam and clonazepam. With this treatment, seizures frequency decreased, but 2-3 episodes/week would still happen despite an increasing of the levatiracetam dose. At 14 years and 6 months she began taking ambroxol at 20mg/Kg/die in 2 doses, then, after 1 year, 25 mg/Kg/die in 3 doses. Frequency of seizures slowly decreased. At the last follow-up date, when the girl was 17, she had only 1 seizure crisis a month, of short duration. Saccadic movements remained pathological. Chitotriosidase fell dramatically from the pre-ambroxol 247 nmol/ml/hour value to 18.16 at the last follow up visit, after 20 months of chaperone treatment.

The second patient is a 31-year-old man, diagnosed with Gaucher disease when he was 4. His genotype was F2131/L444P, a neurologic mutation. He started ERT 10 months after the diagnosis. When he was 11 years old, EEG became abnormal and ocular movements turned out pathologic, with abnormal saccadic movements. When he was 17, myoclonic jerks were observed in conjunction with paroxysmal bouffèes at EEG; a start of a myoclonic epilepsy was supposed and he began sodium valproate therapy. At the age of 23, levatiracetam was added due to a worsening of myoclonus. He remained stable enough, having a generalized seizure episode every 3 years. In the meantime, levatiracetam has been added. When he was 29 he had 3 generalized seizures episodes in 3 months; at that time he started taking Ambroxol at 25 mg/Kg/die in 3 times; right afterwards, EEG showed a diffuse slowing while saccadic movements remained pathological. After 1 month of ambroxol therapy chitotriosidase has not fallen significantly (from 195 to 117.3 nmol/ml/hour). At present, after 1 year of ambroxol therapy he doesn't suffer from generalized seizures.

Conclusion: ambroxol given at high dose (25 mg/Kg/day), as previously shown (Narita et al) seems to improve seizures frequency in GD3 patients. At this point, a trial is necessary to assess the real efficacy of ambroxol on several neurological aspects of GD3.

Narita et al (2016) Ann Clin Transl Neurol 3:200-15

P-10

Very long-term bone mineral density response in a cohort of Gaucher patients treated with ERT from childhood to adulthood

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Osteopenia is described as a relevant sign of bone involvement in Gaucher disease (GD), both in pediatric and adult patients. In a previous retrospective study in a cohort of 18 pediatric patients (1), we observed that after a long-term follow up (4-17 years), only 2 of them (male and female siblings who underwent splenectomy at the age of 3 and 4 respectively) developed a pathologic bone mineral density (BMD) with a Z-score ≤ -2 (2). At the end of this study, 11 patients reached adulthood (≥ 18 years old) and 7 were still in pediatric age.

All the patients were then followed for a further period of 6 to 10 years. At the end of this second study we were able to collect data from 15 adult and 1 pediatric patients; 2 patients were lost at follow-up. The obtained results showed a reduction of BMD Z-score in 8 patients and an increase in 7 of them. The value remained pathologic only in the 2 siblings, respectively -2.0 in the male and -1.9 in the female. We also analyzed the BMD Z-score in patients who reached bone peak mass (aged ≥ 25) (3). Again pathological data were found only in the 2 siblings.

Conclusion: the prolonged follow-up of our cohort, that included early adulthood, did not show a significant modification of BMD bone mineralization compared to data referring to pubertal spurt. Moreover, a precocious splenectomy and a delayed initiation of enzyme replacement therapy showed to be a risk factor to develop a pathologic BMD Z-score and a final reduction of the mass bone peak. The study points out the need for a shared definition of pathologic BMD value both in pediatric and adult population (age ≤.50 in male and pre-menopausal period in female)). Our study indeed shows that osteopenia in Gaucher's disease is less relevant than generally reported. The improving of diagnostic capacity observed in Gaucher disease during the last two decades and the appropriate beginning of ERT have dramatically reduced the probabilities to find severe clinical phenotypes such as the one presenting in our siblings.

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P-11

Genetically modulated Substrate Reduction Therapy for Mucopolysaccharidoses – in vitro studies

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Mucopolysaccharidoses (MPSs) are caused by dysfunction in enzymes responsible for the intralysosomal degradation of glycosaminoglycans (GAGs). We have designed an RNA-based strategy based upon the selective downregulation of genes involved in the biosynthesis of GAGs, which is currently under evaluation. Our goal is to promote an effective reduction of the accumulating substrate, ultimately decreasing or delaying MPSs' symptoms.

Taking advantage of the RNA interference (RNAi) technology potential, we have designed and assayed specific siRNAs targeting genes on those biosynthetic cascades to decrease the levels of production of each one of the four substrates: dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), and chondroitin sulphate (CS). MPSs were divided into two major sub-groups: (1) those that accumulate DS/CS and (2) those that accumulate HS. 'Group 1' included MPS types I, II, VI and VII, while 'group 2' includes the Sanfilippo syndrome, or MPS III, which subdivides into four different diseases: IIIA, IIIB, IIIC and IIID. Proof of principle on the effect of siRNAs targeting CHSY1 and XYLT1 was achieved for two independent control cell lines, with 8-12 fold decreases on the target mRNA levels, after 24h of incubation with concentrations of each siRNA as low as 20nM. Subsequent analysis on the effect of those same siRNAs on patients' cell lines resulted in significant CHSY1 expression decrease in MPS I/MPS VI cell lines ('group 1'), as well as that of XYLT1 in MPS IIIA and IIID fibroblasts ('group 2'). Initial studies evaluated mRNA levels after 24-48h incubation with each siRNA. Even though relevant decreases were observed for all tested cell lines, it became evident that the treatment efficacy may depend on the features of each specific MPS cell line, with some lines requiring higher siRNA concentrations to promote similar inhibition levels.

In order to assess the effect of that treatment on substrate reduction, we have used both the routine Alcian blue and a modified, more sensitive 1,9-dimethylmethylene blue assay on the culture media collected after seeding and incubation, at different time points. Nevertheless, the low confluency levels required for siRNA transfection did not allow detection of GAGs excreted to the culture media. Similar problems have been noted by other authors, particularly in small samples, like the ones we used. Thus, we are currently quantifying GAGs' storage by direct measurement of tissue samples after papain extraction. By doing so, we can access the intralysosomal levels of GAGs instead of their excretion.

Here we present an overview of the preliminary results of this project and unveil its next steps towards a full characterization/evaluation of its potential therapeutic effect.

P-12

Niemann Pick type C in Italy: an update of molecular and biochemical data

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Niemann Pick type C (NPC) is an autosomal recessive lysosomal storage disorder caused by mutations in *NPC1* or *NPC2* genes. In 2009, the results of the molecular characterisation of 44 Italian patients affected by this disorder have been published (Fancello et al., 2009). Since then, 32 NPC patients belonging to 27 families, have been identified in our Centre. Here, we present an update of the biochemical and molecular results obtained in NPC patients identified so far.

NPC1 and *NPC2* coding region were sequenced in all cases. MLPA analysis and RT-PCR studies of the mRNA extracted from cells treated with anisomycin, were performed whenever possible. Overall, 76 patients, belonging to 61 families (57 NPC1 and 4 NPC2) were analysed. Eight patients (5 NPC1 and 3 NPC2) presented the neonatal phenotype, 34 patients showed the early (17) and late (17) infantile phenotype, 14 presented the juvenile form and 15 the adult form (13 NPC1 and 2 NPC2); 5 patients remain unclassified.

Fifty-six different *NPC1* and 4 *NPC2* alleles were identified, all of them due to point mutations. All NPC2 mutations were identified in homozygosity. The most frequent NPC1 mutation was the c.852delT accounting for only 6.5 % of the alleles.The common p.I1061T mutation, associated with the juvenile clinical form, presented an allele frequency of 4.4%, confirming that this variant is much less frequent in Southern than in Northern Europe. Interestingly, one patient carrying this mutation presented the severe neonatal phenotype.

Filipin staining was available for 43 patients, all but 5 presented a classical biochemical phenotype. Among patients with the variant phenotype, one carried the p.P1007A *NPC1* mutation, while 3 presented the p.N222S *NPC1* mutations in compound heterozygous. Although the pathogenic nature of this variant is controversial, all patients presented clinical features of the disease and 2 of them displayed elevated levels of oxysterols in plasma.

Among the new diagnosed patients, 6 novel *NPC1* mutations were identified, 5 missense and one deep intronic mutation that creates a new donor splice site within intron 23, leading to the partial retention of the intron within the mature mRNA, the generation of a premature stop codon and a degradation of the transcript by NMD.
It is worth noting that 11 (9 NPC1 and 2 NPC2) out of the 32 newly diagnosed patients presented the adult form of the disease, while only 4/44 with this phenotype were reported in our previous work, suggesting an increase in the rate of diagnosis of patients presenting less severe, and probably under diagnosed, forms of the disease.
In conclusion, taken together our previous published data and new results, we provide an overall picture of the biochemical and molecular characteristics of NPC patients in Italy.

P-13

Among accumulated Heparan sulfate oligosaccharides, hexasaccharides are the most pathogenic fractions involved in glia activation in Sanfilippo syndrome

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Mucopolysaccharidosis (MPS) type III is a genetic deficiency of enzymes involved in heparan sulfate hydrolysis, inducing accumulation of partially degraded heparan sulfate (HS) oligosaccharides in tissues. HSO chains are multimers of disaccharide units composed of an aminosugar (N-acetylglucosamine) and an uronic acid (glucuronic or iduronic acid) with variable substitutions and different degree of sulfatation.

Accumulation of HSO in central nervous system (CNS) triggers neuroinflammation, oxidative stress and neurodegeneration. Our main objective is to explore the mechanisms by which HSO induce these CNS alterations in MPS III.

We isolated HSO from MPS III urine patients using gel electrophoresis and we analysed their structures by MALDI-TOF/MS. We then identified different HSO polymerization degrees (PD) ranging from di- to dodeca-saccharides. These different PD fractions were reproduced *in vitro* by enzymatic heparinase HS digestion and separated by size exclusion chromatography. Each PD fraction was then added to microglia, astrocyte and neuron cultures. Among the different PD of HSO, we showed that only hexasaccharides activate the production of pro-inflammatory cytokines and increase anti-oxidant enzyme expression when added to microglia or astrocyte cultures. However, these fractions have no effect on neuronal cells. Interestingly, transcriptomic studies showed increased expression of genes related to oxidative stress, iron metabolism and inflammatory pathway only in microglia treated with hexasaccharides and not when treated with other PD fractions. We conclude that, among accumulated HSO, hexasaccharides are the most pathogenic fractions. Targeting this specific hexasaccharide fraction opens new therapeutic approach in Sanfilippo syndrome.

P-14

Early hematopoietic stem cell transplantation in a MPS type VII boy

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Sly disease (mucopolysaccharidosis (MPS) type VII, OMIM #253220) is a rare autosomal recessive lysosomal storage disorder due to deficient activity of beta-glucuronidase leading to accumulation of glucuronic-acid containing glycosaminoglycans. The disease shows considerable phenotypic variability, ranging from a severe antenatal form with non-immune hydrops fetalis to mild adult forms [1]. Here we report the case of a boy, born in Feb. 2015 to non-consanguineous parents, who presented at 9 days with lymphedema, coarse facies, club foot (*talipes equinovarus*) and slight hepatosplenomegaly. Vacuolated leukocytes were seen on a blood smear. Traces of dermatan sulfate were found in the urine along with a marked enzyme deficiency of both leukocyte and plasma beta-glucuronidase. Sanger sequencing of the *GUSB* cDNA evidenced 3 missense variations, all being potentially pathogenic (two were novel). When the patient was 2-months-old, kyphosis was noted while psychomotor development appeared normal. Enzyme replacement therapy using UX003 (Ultragenyx) [2] was started in June 2015, which was well tolerated and led to a reduction of visceromegaly and skin infiltration. Stem cell allograft transplantation (HSCT) was done when the patient was 13-months-old. After a severe skin and gut GVH disease, enzyme replacement therapy was stopped 6 months after HSCT. The last follow-up examination (at the age of 28 months) revealed a slow-down of the growth curve, persistent kyphosis, no hepatosplenomegaly, and no other organ involvement. Enzyme activity has normalized in leukocytes but remains low in plasma. This case report illustrates i) the need for an early diagnosis of MPS, and ii) the possible benefit of a very early enzymatic and/or cellular therapy in this rare form of lysosomal storage disease.

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P-15

Is acid glucosylceramidase a player in the development of cutaneous melanoma?

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Cutaneous melanoma (CM) is a severe type of skin malignancy responsible for the majority of skin cancer deaths. In spite of recent targeted therapies like BRAF inhibitors, the prognosis of patients with advanced CM remains poor. Several studies, including from our group, have shown alterations of sphingolipid, especially ceramide metabolism in CM. Analysis of the exome of patients with CM revealed missense mutations of the *GBA* gene. *GBA* encodes acid glucosylceramidase (GCase), a lysosomal hydrolase whose deficiency of the enzymatic activity results in Gaucher disease [1]. Of note, the mutations found in CM patients also lead, at the homozygous state, to Gaucher disease. Interestingly, patients with Gaucher disease have an increased risk for the development of malignancies, including CM; the underlying molecular bases of this risk are presently unknown. Here, we aimed at determining the role of (wild-type and mutant) GCase in melanoma cell growth and response to treatment.

To do this, we have created several cellular models: one in which GCase is overexpressed by lentivector transduction of human A375 melanoma cells, and two others in which GCase expression and/or activity is inhibited either by shRNA against *GBA* or by a pharmacological inhibitor. Viability assays on these different cellular models showed no differences in the sensitivity of melanoma cells to dabrafenib, a BRAF inhibitor. However, whereas only small differences were noticed in two-dimensional culture, three-dimensional agar culture assays showed that clones with reduced GCase expression formed loosened aggregates that were less delimited than the spheroids formed by control clones [1]. Finally, colony forming assays revealed that cells overexpressing wild-type or even mutant GCase formed more colonies than control cells.

These results suggest that (wild-type or mutant) GCase does not critically influence the response to targeted therapy but may affect clonogenic and migratory abilities. This effect might be independent of the catalytic function of GCase but rather possibly due to a gain-of-function of the protein.

Supported by INSERM, Ligue Nationale contre le Cancer and VML.

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P-16

Newborn screening for Fabry disease in the Italian regions of Tuscany and Umbria: current overview

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Background: In November 2014, a newborn screening pilot project for Fabry disease (FD) was introduced in Tuscany and Umbria, two Italian regions where an expanded newborn screening (NBS) programme has been in place since 2004.
FD is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal enzyme alpha-galactosidase A (α-Gal A) due to mutation of the GLA gene. FD is characterized by a heterogeneous spectrum of clinical presentations and includes the severe classic male phenotype, later-onset phenotypes and variable clinical presentations in female patients ranging from asymptomatic to the severe classic phenotype. Disease progression may result in renal, cardiac and neurological disease with severe morbidity and reduced life expectancy. Specific enzyme replacement therapy and chaperone therapy are available for FD. Early detection allows higher treatment efficacy preventing the development of irreversible organ damage.

Aim: To estimate the prevalence of FD among the analyzed newborn population, to allow early diagnosis and to extend the study to at risk family members thus avoiding diagnostic delays and providing genotype-phenotype correlation of novel variants.

Methods: α-Gal A enzyme assay was carried out on the same DBS used for expanded NBS. Newborns with low α-Gal A enzyme activity (≤2.38 μmol/l/h) were retested and confirmed by GLA gene sequencing.

Results: Up to May 2017, 52 592 newborns were screened. 19 resulted positive for the α-Gal A enzyme assay, 14/19 were confirmed by α-Gal A enzyme assay on leukocytes and by GLA gene sequencing (PPV% 73.7). By genotyping, we identified 2 known mutations related to classic FD and 3 known mutations reported in late onset FD. 2 further GLA variants were reported but with unclear clinical significance. 1 GLA variant was novel. By pedigree analysis we found that all mothers were heterozygous and we detected a further 7 additional relatives carrying a GLA gene mutation. In two families, the grandfathers died prematurely without diagnosis presenting cardiomyopathy and/or renal failure, another grandfather presented late onset renal involvement.

Conclusions: The estimated incidence of FD in Tuscany and Umbria among the newborn population is 1 : 3756. All patients identified by NBS are in long-term follow-up to assess when they need to start treatment. Pedigree construction is a useful tool for diagnosing FD patients among at risk family members, particularly heterozygous females so that we can provide accurate genetic counselling and early treatment.

P-17

Strategies compared: diagnostic next generation sequencing (NGS) and biochemical markers of NPC in at risk populations.

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Clinical presentation of Niemann-Pick type C (NPC) is highly variable and patients with non-classical forms of NPC have been found in increasing numbers in patients with largely non-specific neurological symptoms, psychiatric symptoms, isolated organomegaly or a combination of the above. Recent availability of disease-modifying treatments makes timely diagnosis of NPC more urgent than before.

We have evaluated approaches to detection of NPC in at risk populations on the basis of experience with two diagnostic tests. First, we used next generation sequencing(NGS) of 3000+ genes panel that contained the majority of known disease-causing genes including NPC1 and NPC2 in 50 patients (age range 2-66y, average 18.6y, median 11y) with neurological symptoms, in whom previous clinical, imaging and biochemical testing did not lead to diagnosis, although in some of them there was a suspicion for a group of disorders. Partial data were reported at ESGLD 2015. NGS identified variants that led to diagnosis in 12 patients (NCL7 - 2x, NCL8, Lafora disease – NHĹRC1, Cbl J – ABCD4, CDGIIh - COG8 2x, AD mental retardation 5 – SYNGAP1, AD mental retardation 6 – GRIN2B, X-linked dyskeratosis – DKS1, AD Noonan syndrome 4 – SOS1, partial biotinidase deficiency - BTD), possibly pathogenic variants, where the diagnosis remained unconfirmed in further 10 patients, and variants that were possibly pathogenic, but apparently not related to symptoms, in 10 patients. While lysosomal disorders (NCL7, NCL8, Cbl J) were found in the cohort, no NPC patients were detected.

We have also tested second generation NPC biochemical markers lysosphingomyelin and lysosphingomyelin-509 in dried blood spots (DBS) and plasma from NPC patients, NPA/B patients, and controls. Combined testing of both markers allowed for good separation of all three groups in plasma, but in DBS it was not possible to distinguish reliably patients with NPC and NP A/B (Kuchař et al., 2017).

In a small cohort (n=50) of predominantly younger patients (age median = 11y) with unexplained neurological disease no NPC patients were found; however, NPC patients were previously detected from the same population on the basis clinical suspicion. NGS sequencing will likely remain a common diagnostic tool in such patients, although in our group it led to diagnosis in only a minority of patients (24%). Second generation NPC biochemical markers, which have a significant advantage over the first generation markers, will be especially useful in patients with suspicion of NPC and adult patients with unexplained neuropsychiatric disease.

Kuchař L et al (2017) Anal Biochem 525: 73-77

Supported by grant AZV16-33923A/2016 from the Ministry of Health of the Czech Republic

P-18

Investigation of the lysosomal proteome in different nutrient conditions

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The lysosomal protein composition changes depending on nutrient conditions. For example mTORC1 which under nutrient sufficiency is associated with the lysosomal surface dissociates into the cytoplasm under starvation which is accompanied by its inactivation. Thereby the phosphorylation of mTORC1 target proteins is abolished. Similarly, glucose deprivation leads to dissociation of parts of the V-ATPase complex from the lysosomal surface. Given the multiple functions of the lysosome and the complexity of cellular metabolism, it is likely that also other proteins change their localization or expression dependent on the cell's nutritional status.

The objective of our study is to get a comprehensive overview about the lysosomal proteome and phoshoproteome in different nutrient conditions. HEK 293 cells were cultured under normal conditions, starved and starved followed by refeeding. Lysosomes were isolated from these cells via a magnetic bead based lysosome isolation technique. Using different markers for lysosomes, endoplasmic reticulum and mitochondria. We show that the final fraction is highly enriched in lysosomes. The cells exposed to the three different conditions (control, starved, starved+refed) were labelled with light, medium or heavy stably isotope labelled amino acids (SILAC). We compared datasets of lysosomal fractions of untreated cells versus cells starved for 50 min (data set 2) and untreated versus 120 min starved/30 minutes readdition of medium (data set 1). In data set 1 we found 47 proteins upregulated by at a factor of 1.5 and 15 proteins downregulated by at least 50%, respectively. Since in data set 2 only very few proteins were found when the same criteria were applied we decreased the values for up or downregulation to 1.25 or 0.75 fold, respectively. This revealed 76 proteins upregulated and 42 downregulated in data set 2, respectively. To identify most promising candidates we applied Venn diagrams for proteins which were either up- or downregulated by the respective factors. The Venn diagram identified only 2 proteins (Dynactin and pyruvate kinase) upregulated in both data sets. In contrast the Venn diagram identified 12 proteins downregulated in data sets 1 and 2. Among these were the raptor subunit of the mTORC1 complex, mTOR kinase itself, and 4 subunits of the V-ATPase. All these proteins have been described to dissociate from the lysosome upon starvation and thus prove the reliability of the data sets. In all data sets we identified five additional proteins which were consistently downregulated, one of which is the eukaryotic translation factor 4E (eIF4E). We could verify by Western Blot analysis that eIF4E is located on the lysosomal surface and that it dissociates during starvation. The physiological function of this phenomenon is still unclear.

P-19

Chitotriosidase activity and expression in mice with lipid storage syndrome treated by macrophage stimulator

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Lipid storage is common in some lysosomal diseases, liver steatosis and atherosclerosis. Polysaccharides (β-glucans, mannans) were shown to stimulate macrophages *in vivo* and can be used for effective removing of circulating lipoproteins and improve lipid storage.

The aim: to evaluate the protective effect of mannan *Candida albicans* serotype A (mannan) in a mouse model of hyperlipidaemia and liver lipid storage. Inhibitor of lipase poloxamer 407 (P-407) was used in CBA mice (300 mg/kg). Mannan (Inst. Chemistry, SAS, Slovakia) was administered before P-407 (50 mg/kg).

In vitro mannan (50 μg/mL) increased proliferation of murine peritoneal macrophages, increasing their NO production. *In vivo* pre-treatment by mannan followed by hypolipidemic effect, decreasing accumulation of lipids, increased number of macrophages in liver; decreased number and size of lipid droplets in cytoplasm of hepatocytes was shown.

Mannan caused a significant increase in serum chitotriosidase activity in P-407-induced hyperlipidaemic mice. Hepatic expression of *Chit1* and *Chia1* was shown to increase in liver relative to control following the administration of mannan to both non-hyperlipidaemic and P-407-induced hyperlipidaemic mice. Lipid droplets have been identified as a substrate for macroautophagy; they can be removed after fusion with autophagosomes followed by digestion inside of autophagolysomes (Miyagawa et al., 2016). Increased chitotriosidase activity and *CHIT1* and *Chia1* expression reflects macrophages stimulation in experimental lipid storage syndrome induced by mannan. Understanding of mechanisms how lipophagy clears lipid droplets is the topic for further research.

Miyagawa K et al (2016) Am J Pathol 186: 1861-1873

Supported partially by grant RFBR №16-04-1423

P-20

Activity-based labeling and detection of active lysosomal glycosidases: application in diagnostic screening of urine samples

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Many lysosomal glycosidases are retaining enzymes, employing a double-displacement mechanism involving covalent binding of sugar to the catalytic nucleophile of the enzyme [1]. Cyclophellitol is natural gluco-mimetic that irreversibly binds to the catalytic nucleophile of glucocerebrosidase (lysosomal β-glucosidase; GBA). Based on this scaffold a fluorescent activity-based probe (ABP) was designed allowing labeling of active GBA in vitro and in vivo (MDW933) [2]. Subsequently appropriate ABPs were designed for other lysosomal glycosidases, including acid α-glucosidase (GAA) and α-galactosidase (GLA) [3-5].

The available ABPs can be used in diagnosis of corresponding lysosomal storage disorders. Cells, or cell extracts, can be incubated with ABPs, and following SDS-PAGE and fluorescence scanning active enzyme molecules can be visualized and quantified. In this manner a deficiency of active, GBA, GAA and GLA can be demonstrated in cells from individuals suffering from Gaucher disease, Pompe disease and Fabry disease, respectively [2-5]. We recently also extended the procedure to urine and developed a method to visualize active GBA, GAA and GLA in urine samples. The method is remarkably sensitive, requiring only a few ml of urine. The practical application of this procedure for diagnostic screening is discussed.

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P-21

Development of an antisense-mediated exon skipping approach as a therapeutic option for the ML II-causing mutation c.3503_3504delTC

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Lysosomal Storage Disorders (LSDs) are a group of rare inherited diseases caused by the malfunction of the lysosomal system, resulting in the accumulation of undegraded substrates inside the lysosomes and leading to severe and progressive pathology. Among them is ML II, one of most severe LSDs, which is caused by the total or near total deficiency of the GlcNAc-phosphotransferase, a key in enzyme for the correct trafficking of lysosomal hydrolases to the lysosome. GlcNAc-phosphotransferase is a multimeric enzyme and is encoded by two genes: *GNPTAB* and *GNPTG*. One of the most frequent ML II causal mutations is a dinucleotide deletion on exon 19 of the GNPTAB gene that disrupts the reading frame and prevents the production of an active GlcNAc-phosphotransferase, which in turn impairs the proper targeting of lysosomal enzymes. Despite broad understanding of the molecular causes behind this and other LSDs, the same progress has not been observed in the development of new therapies, with current treatments still mostly symptomatic and presenting several limitations. Therefore, alternative options should be investigated in order to provide patients and families with better healthcare and more promising therapies. One possibility is the modulation of splicing by antisense oligonucleotides with the purpose of altering the splicing pattern, the mature mRNA and ultimately the final protein product. Acknowledging this, the present study intends to design and develop a RNA-based therapeutic agent through the use of antisense oligonucleotides capable of inducing the skipping of exon 19 of the GNPTAB gene and consequently circumvent the effects the most common ML II causal mutation. The approach is presently ongoing and different 2'O-Methyl AOs were designed and synthesized to target the GNPTAB exon 19 and promote its skipping. We have already succeed in inducing the skipping of exon 19 in control and ML II patient cell lines and we are now evaluating the effects of this therapeutic approach at biochemical levels.

P-22

Anti-agalsidase antibodies associated with renal transplantation in Fabry disease

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Fabry disease is a X-linked disease in which *GLA* mutations lead to alpha-galactosidase A deficiency with subsequent increase of glycosphingolipids such as *Lyso*Gb3. Enzyme replacement therapy (ERT) with agalsidase brought benefits when introduced at the early stages of the disease but anti-agalsidase antibodies of unclear significance can develop. We aimed to determine the clinical and biological impact of these antibodies.

From December 2014 to January 2017 we prospectively screened patients with genetic and/or enzymatic diagnosis of Fabry disease from our multicenter collaborative cohort (FFABRY) for anti-agalsidase antibodies with a home-made ELISA, neutralization tests and plasma Lyso-Gb3 then compared with clinical outcomes.

Among the 103 patients included (53 men), 18 of the 45 men (40.0%) and 2/25 (8.0%) of women exposed to agalsidase were seropositive without difference between molecules. Antibody titers were correlated with enzymatic inhibition (r^2>0.77, p<0.0001) and plasma *lyso*Gb3 in men (r^2=0,52; p<0.0001). All IgG subclasses were observed but IgG4 reached the highest concentrations (0.05 to 1.45 mg/ml). Seropositivity, but not serum inhibition was associated with higher frequencies of renal transplantation or dialysis (hazard ratio 5.93; p=0.014) independently from the age, angiotensin-conversion-enzyme inhibitor exposure, mutation and delay of agalsidase treatment. Mutations leading to truncated alpha-galactosidase A were significantly associated with antibodies (relative risk 2.88; p=0.006) in men and correlated with worse cardiac MSSI in women.

Whereas the role of the intrinsic alpha-galactosidase production by the renal graft remains to be elucidated, the association observed with anti-agalsidase antibodies can illustrate an unexpected effect of transplantation despite immunosuppressants or at least make anti-agalsidase antibodies a marker for renal severity in Fabry disease. Systematic screening for antibodies should be performed after transplantation or ERT introduction. A better understanding of the role of genotype in antibody development is needed. New and more aggressive therapeutical algorithm could already be discussed to dampen antibodies effect by increasing the dose of ERT, associate chaperone therapy to hide epitopes or use immunosuppressive therapies.

P-23

Treating neuronal proteostasis in lysosomal storage diseases

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Understanding mechanisms by which lysosomal storage and dysfunction leads over time to neuropathology makes it possible to develop new therapies for lysosomal storage disorders (LSDs). Maintaining protein homeostasis (proteostasis) is important to preserve cellular viability and functions. This is particularly true for neurons, which are post-mitotic cells. By studying different mouse models of LSDs, including MPS-IIIA, we found that amyloid aggregates massively accumulated into neurons as consequence of impaired degradation capability of cells. Qualitative and quantitative analysis based on Congo Red and Thioflavin stainings revealed that such aggregation is progressive and mainly localized to neuronal bodies. Moreover, amyloid deposition is more pronounced in specific central nervous system (CNS) regions such as neocortex, striatum and pons. Characterization of amyloid inclusions showed a heterogeneous composition, which also change depending on the CNS region analyzed. Importantly, some disease-relevant proteins including α-synuclein are predominant component of these aggregates. Our data suggest that amyloid aggregation may play a key role in LSD neuropathogenic processes not only by inducing neurotoxicity associated to amyloid aggregation itself but also by loss of function mechanisms relying on the sequestration of key proteins and the consequent prevention of their biological functions. We are now further characterizing amyloid aggregates focusing on both their composition and neuro-pathological pathways induced. Moreover, we are also exploring the possibility to slow down disease progression in LSDs by treatment with compounds able to destroy amyloid aggregation.

P-24

Early onset gait abnormalities and spinal cord pathology in a mouse model of CLN1 Disease

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The neuronal ceroid lipofuscinoses (NCLs) are a group of up to 14 progressive neurodegenerative lysosomal storage disorders that mainly affect children and young adults. All forms are fatal after a period of progressive disability. Infantile NCL (INCL, CLN1 Disease) is an early onset and rapidly progressive form of NCL caused by a deficiency of the lysosomal enzyme palmitoyl protein thioesterase 1 (PPT1). Symptoms include loss of vision, epileptic seizures and loss of cognitive and sensorimotor function. The disease is characterized in both humans and PPT1-deficient (*Ppt1*$^{-/-}$) mice by significant pathology in the brain and cerebellum, including progressive glial activation, loss of different neuron populations, synaptic pathology and the accumulation of autofluorescent storage material. So far, the motor deficits seen in *Ppt1*$^{-/-}$ mice have been attributed to cerebellar defects, but spinal pathology that contributes to clinical outcome has recently been described in CLN1 disease. To investigate the nature of motor defects in this disorder in more detail we characterized *Ppt1*$^{-/-}$ mice using the *CatWalk XT* gait analysis system at monthly intervals. This revealed unexpectedly early gait abnormalities, including an initial period of hypermobility followed by a progressive loss of motor function. A comprehensive histopathological characterization of spinal cord pathology in *Ppt1*$^{-/-}$ mice, revealed that profound pathological changes occur throughout the spinal cord unexpectedly early in disease progression, before the onset of similar changes in either the brain or cerebellum. There were also novel spinal cord-specific phenotypes including a developmental volumetric deficit and pronounced white matter pathology, which are not observed in other parts of the CNS. The successive loss of different spinal neuron populations in *Ppt1*$^{-/-}$ mice may explain the nature of early gait abnormalities in these mice. Given that the spinal cord has largely been overlooked as a site of pathology in any form of NCL, these novel and unexpected data have important implications for this disorder. These data not only fundamentally change our understanding of disease pathogenesis, but will also impact the timing and targeting of future therapeutic approaches in this as well as other forms of NCL, in which spinal pathology also appears to be present.

P-25

CSF Lysosomal Enzymes Activity and GBA1 Genotyping in Parkinson's Disease

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Lysosomal dysfunctions are a prominent pathogenic feature of Parkinson's Disease (PD). Mutations in the *GBA1* gene encoding the lysosomal enzyme beta-glucocerebrosidase (GCAse) are the most common risk factor for PD. GCase activity has been found significantly reduced in brain and in cerebrospinal fluid (CSF) of PD patients with and without *GBA1* mutations. Additionally, in CSF of PD patients, the activity of other lysosomal enzymes has been found altered. Here we investigated the activity of the lysosomal enzymes GCase, cathepsin D (CatD), cathepsin E (CatE) and beta-hexosaminidase (b-hex) in CSF of 71 PD patients and 69 healthy controls (CTRL) from the BioFIND cohort. Our aims were to confirm the role of CSF GCase activity as Parkinson's disease biomarker, also considering the presence or not of GBA1 mutations and to assess the CSF activity of other lysosomal enzymes as possible PD biomarkers. The obtained results show that GCase and b-hex specific activities (SA) were significantly associated with a worse cognitive performance . Of interest, according to H&Y score (H&Y<2 and H&Y≥2) a significant decrease in GCase and CatD SA was observed in the more advanced stages of disease. No differences in lysosomal enzyme SA were found between the different PD subtypes. GCase and CatD SA fairly discriminated PD from CTRL when these two enzymes were considered as single parameters (AUC=0.72 and AUC=0.68, respectively). The panel of all the measured lysosomal enzymes SA, led to a better diagnostic performance (AUC=0.77). The inclusion of a-synuclein and Ab1-42 to the model further increased the diagnostic accuracy (AUC= 0.83, 95% CI=0.66-0.88, Sens.= 84%, Spec.= 75%). Mutations in *GBA1* gene were present in 10 out of 79 PD and 3 out of 61 CTR. The c.-203A>G polymorphic variant, located in the *GBA1* promoter region, was also found in 2 PD and 1 CTRL. *GBA1* mutation carriers showed a lower CSF GCase SA with respect to non-carriers (-27%, p=0.042). In PD patients, GCase activity was significantly decreased in *GBA1* mutation carriers compared to non-carriers, as well. However, a significant reduction of GCase SA in PD patients was found even after exclusion of subjects carrying *GBA1* variants (-25%, p<0.001).These findings confirm the potential diagnostic role of CSF GCase activity in PD, also showing the involvement of other lysosomal enzymes (e.g. CatD). CSF GCase activity is lower in GBA1 mutation carriers, however its overall decrease in PD patients is independent of the presence of GBA1 mutations, as previously shown in PD brain. CSF GCase activity is associated with cognitive impairmentCSF GCase and CatD activities are lower in the more advanced stages, suggesting their possible role as PD prognostic markers.

P-26

Cross–regulation of *CLN5* and *CLN6* gene expression in ovine Batten disease models

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Sheep with naturally occurring CLN5 and CLN6 forms of Batten disease (neuronal ceroid lipofuscinoses, NCLs) are studied as models of the human diseases. Despite having mutations in different genes, the development of clinical and neuropathological disease in CLN5 and CLN6 affected sheep is very similar. Common features, including the near-ubiquitous accumulation of lysosome-derived storage bodies, regional brain atrophy from progressive neuronal loss, retinal degeneration, seizures and psychomotor decline culminating in premature death follow a very similar time-course in both forms. This is unexpected as the *CLN5* and *CLN6* genes and mutations are quite different and apparently unrelated. CLN5 disease is caused by an intronic splice-site mutation resulting in excision of an exon from the mRNA for a soluble lysosomal protein, while CLN6 results from a large 5'UTR-exon 1 deletion from the gene for a membrane bound protein of unknown function.

Antibodies specific for the ovine CLN5 protein revealed a band of concentrated endogenous staining in the hippocampus of unaffected control sheep, that was not apparent in CLN5 affected sheep. Surprisingly there was very little CLN5 expression in CLN6 affected sheep either, and only intermediate staining in heterozygous $CLN6^{+/-}$ sheep. The implied interaction of CLN5 and CLN6 expression was explored further in a quantitative PCR study of brain samples. Full length CLN5 mRNA expression was up-regulated 3-4 fold in CLN6 affected brains, thus there is plenty of CLN5 mRNA but this does not result in successful CLN5 protein expression. Heterozygotes gave intermediate values. Similar results were obtained from other non-neuronal tissues, showing that they did not arise from neurodegeneration. Control CLN6 expression was 10 fold less than CLN5 expression and was up-regulated in some brain regions in CLN5 affected sheep.

These results suggest that the CLN5 and CLN6 protein and gene expressions are cross-regulated. CLN6 is an endoplasmic reticulum resident protein. If it is required for the correct processing of the CLN5 protein, then it may be that no CLN5 protein reaches the lysosome in CLN6 affected sheep, resulting in an induced lysosomal CLN5 deficiency disease. CLN5 transcription may be up-regulated in a vain attempt to rectify this. The CLN6 protein expression may become up-regulated likewise if it receives no substrate.

P-27

Plasmatic biomarkers for the screening of Niemann-Pick type C disease: experience in a clinical setting in France

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The diagnosis of Niemann-Pick type C (NPC) disease has long relied on time-consuming filipin staining test on fibroblasts, associated with genetic study of *NPC1* and *NPC2* genes, sometimes difficult to interpret. The emergence of sensitive plasmatic biomarkers in the early 2010's, i.e. oxysterols and the analogue 509 of lysosphingomyelin (LysoSM509), has changed the paradigm of NPC diagnosis and now allows an easier and more systematic screening of this disease.

Plasmatic oxysterols (cholestane-3β,5α,6β-triol and 7-ketocholesterol) and lyso-sphingolipids panel measurement by LC-MS/MS (including LysoSM509) have been implemented in our laboratory. We report here our current experience on analysis of these biomarkers for NPC diagnosis.

Biomarkers measurement was performed for 325 patients referred to our laboratory in 2016 for NPC screening purpose (compared to approximately 100 filipin staining tests/year in previous years), including adults (75%) and children (25%). Twenty-five patients displayed pathological levels for at least one marker (oxysterols and/or lysoSM509, and/or lysoSM). Depending on the biomarker profile, suitable complementary investigations were completed for 22 patients (in progress for the remaining 3). Following filipin staining and/or gene sequencing, 10 patients were confirmed as NPC index cases (8 children and 2 adults), and 3 classified as NPC heterozygotes. Five patients with combined striking elevation of lysoSM were confirmed as suffering from sphingomyelinase deficient Niemann-Pick disease, and one adult patient with oxysterols elevation only, from cerebrotendinous xanthomatosis. Only 3 cases currently remain as unresolved false-positives and no false negative has been encountered so far.

The implementation of plasmatic biomarkers as the first-line test for NPC now allows a large-scale screening of this disease, especially in adult patients with incomplete clinical picture. A confirmation of the diagnosis is always mandatory. The French national recommendations for NPC diagnosis strategy are now as follows: (i) biomarkers measurement as the first line step (ii) *NPC1* and *NPC2* gene sequencing as the confirmation step (iii) filipin staining test for difficult cases (e.g. patients with only one mutation identified, patients with variants of unknown significance, patient's parents not available for genetic study), or for some rare unsolved cases with strong clinical suspicion. The combination of oxysterols and lysosphingolipids multiplex measurement allows the differential diagnosis of other disorders associated with increase of either biomarker.

P-28

Suppressed autophagy in a mouse model of neurodegeneration and autophagy stimulation in brain by rapamycin and trehalose

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Lysosomal diseases are connected mainly with neurodegenerative disorders, usually as result of accumulation of toxic proteins and damaged organelles. Autophagy insufficiency occurs in various neurodegenerative pathologies and in ageing. So, autophagy activation is regarded as a promising tool for treatment of neurodegeneration.

Normal mice of C57Bl/6j strain and a mouse model of Parkinson's disease (B6.Cg-Tg(Prnp-SNCA*A53T)23Mkle/J strain) were used. Autophagy was evaluated by immunohistochemistry of LC3-II, osmotic test on autophagy and segregation of LDH into vesicles (Seglen et al., 2015). Dopaminergic neurons and their fibers in the striatum and s.nigra (midbrain) were studied.

According to detection by LC3-II expression, autophagy was considerably suppressed in the striatum and to a lesser degree in s.nigra of 5-month old B6.Cg-Tg(Prnp-SNCA*A53T)23Mkle/J mice compared to the control C57Bl/6 mice. The marker level was much higher in the frontal cortex than in nigrostriatum. For stimulation of autophagy we used rapamycin targeted to the main autophagy regulator mTOR, and a disaccharide trehalose activating autophagy via mTOR-independent way. Autophagy activity in the brain of C57Bl/6 mice estimated by osmotic test was enhanced by both rapamycin (5-10 mg/kg, daily, one week) and trehalose (2 % solution in drinking water). The highest autophagy activation was produced by combined treatment with rapamycin and trehalose. More clear effect was observed in the striatum than in midbrain. New approach for therapeutic stimulation of autophagy in a mouse model of neurodegeneration was suggested.

Reference

Seglen PO et al (2015) Methods 75:25-36.

Note: The work was partially supported by grant №16-04-1423 (RFBR).

P-29

Glycosaminoglycan profile in the Mucopolysaccharidosis type II mouse model at baseline and after 6 weeks treatment with ERT

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Mucopolysaccharidosis type II is a lysosomal storage disorder due to the deficit of the enzyme iduronate 2-sulfatase (IDS) and to the consequent accumulation of heparan (HS) and dermatan (DS) sulfate, with multi-organ involvement. We here characterized uronic acid-bearing glycosaminoglycans (UA-GAGs) profile in different organs (brain, liver, kidney, heart, lung) of the Ids knock-out (Ids-ko) mouse model at 12 weeks of age and after 6 weeks treatment with the human IDS (hIDS) enzyme, by using the capillary electrophoresis-laser induced fluorescence (CE-LIF) technique.

As expected, untreated Ids-ko mice showed a heavy accumulation of total GAGs compared to wild-type (wt), ranging from a 4X increase in lung up to 150X in liver. A deeper analysis of single UA-GAGs (hyaluronic acid, HA; chondroitin sulfate, CS; DS; HS) highlighted that cumulative CS and DS (CS+DS) and, above all, HS contribute to the observed increase in all organs, whereas HA appears slightly increased in the Ids-ko mice only in the kidney (1.2X). The evaluation of the HS chemical groups composition underlined that the 2-*O*-sulfated (2s) species are always slightly increased (1.6X) in the Ids-ko mice, 6-*O*-sulfated (6s) species remain unaltered only in the liver and the *N*-acetyl (Na) reduces slightly in liver and heart. The disaccharide composition of CS-DS was examined, pointing out that in liver, heart, kidney and lung the non-sulfated (C0S) and the 6-sulfated (C6S) disaccharides are reduced in the Ids-ko mice, while the 4-sulfated (C4S) disaccharide is increased. This confirmed that the greatest contribution to CS+DS is given by DS, naturally more sulfated in position 4. Differently, in the brain the C4S remains unchanged, the C0S is decreased and the C6S is increased, indicating a secondary accumulation of CS in the Ids-ko mice, possibly suggesting an involvement of the molecule in the neurological pathology.

We conducted the same analysis in Ids-ko mice treated with 1 mg/kg of hIDS, once a week for 6 weeks. As expected, we observed a huge reduction of CS+DS and HS in all organs (from 1.7X in lung to 16.4X in liver) vs untreated Ids-ko mice. Only in the brain we did not observe a reduction of the different UA-GAGs, confirming hIDS inability to cross the blood-brain barrier; only a slight increase in HA levels was observed following treatment.

These preliminary data pave the way for a clearer understanding of the involvement of different UA-GAGs species in the pathology of MPS II and underline the potential of CE-LIF analysis, as a more sensitive technique, for monitoring the therapeutic efficacy. This

becomes particularly important in the brain, where very low GAG levels can be detected by common biochemical techniques.

Funded by the Italian Ministry of Research, PRIN Prot. 20122EK9SZ_003

P-30

Enzymatic method for the determination of the non-lysosomal glucosylceramidase

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Background: The GBA2 gene encodes an enzyme with beta-glucosidase activity that cleavage the bile acid 3-O-glucosides and is also involved in the metabolism of sphingolipids by the reversible hydrolysis of glucosylceramide into free glucose and ceramide. The protein is called non-lysosomal glucosylceramidase to differentiate it from the lysosomal isoform GBA1 that is defective in Gaucher disease. Mutations in the GBA2 gene are responsible for motor neuron defects in hereditary spastisc paraplegia and it was also causative of spastic cerebellar ataxia. More recently, GBA2 was identified as the gene mutated in two families with Marinesco-Sjögren like syndrome, characterized by the triad: cerebellar ataxia, cataracts and progressive muscle weakness due to myopathy.

Objective: The aim of this work was the design of a robust GBA2 enzyme activity assay to be used as a diagnostic tool in identifying GBA2 enzyme deficiency.

Methods: The analysis is performed in leukocytes using the synthetic substrate 4-methylumbelliferyl-beta-D-glucopyranoside in citrate-phosphate buffer (pH 5,8) and AMP-deoxynojirimycin that strongly inhibits GBA2, but not to the same extent GBA1 nor GBA3. The GBA 2 activity is then obtained by subtraction of the fluorescent rate with and without the inhibitor. Since the enzyme is rather unstable the disruption of cells were optimized with a combination of mechanical homogenization followed by soft sonication at 4° C and the enzyme analysis is performed out within 3 hours.

Results: The GBA2 activity in 15 normal controls showed a mean value of 2,7 (1,2 – 4,3) µkatal/kg protein. A patient presenting Marinesco-Sjögren-like syndrome with a homozygous 2bp deletion in the GBA2 gene (c.1528_1529del, located in exon 9) and elevated concentrations of glucosylceramide in erythrocytes and plasma, showed reduced activity of GBA2 (0.2 µkatal/kg protein) corresponding to a residual activity of 7%.

Conclusion: The new enzymatic assay using the GBA2 inhibitor AMP-deoxynojirimycin to minimize interference of beta-glucosidase and glucocerebrosidase activity in leucocytes is adequate to confirm the residual activity of non-lysosomal glucosylceramidase in patients with mutations in the GBA2 gene.

P-31

Characterisation of the phosphatidylinositol(4,5)bisphosphate 4-phosphatase TMEM55A and TMEM55B

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Phosphatidylinositolphosphates (PtdInsP) are a family of low abundant, short lived lipid molecules that play critical roles in the homeostasis of cellular membrane transport. Phosphorylation and dephosphorylation of PtdInsP are transient and highly dynamically regulated processes mediated by both lipid-phosphatases and -kinases. These enzymes are essential for the regulation of central downstream events like fusion or fission of membranes and cell signaling. PtdIns(4,5)P_2 is enriched at the plasma membrane, but there is increasing evidence suggesting an additional role for PtdIns(4,5)P_2 in the regulation of trafficking events and functional processes in non-plasma membrane organelles and particularly in lysosomes.

Two members of the group of PtdInsP-modifying enzymes were previously identified as the only enzymes capable of removing specifically phosphate from the D4 position of PtdIns(4,5)P_2, yielding PtdIns(5)P: namely TMEM55A and TMEM55B. They localized to late endosomes and lysosomes and apart from the initial characterisation, very little is known about both phosphatases.

Here we show first data of deciphering the physiological roles of TMEM55A and TMEM55B.

P-32

Lysosomal acid lipase deficiency in 23 Spanish patients: High frequency of the novel c.966+2T>G mutation in Wolman disease.

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Lysosomal acid lipase (LAL) is a lysosomal key enzyme involved in the intracellular hydrolysis of cholesteryl esters and triglycerides. Patients with very low residual LAL activity, present with the infantile severe form Wolman disease (WD), while patients with some residual activity develop the less severe disorder known as Cholesteryl ester storage disorder (CESD). LAL is encoded by *LIPA* gene. We present the clinical, biochemical and molecular findings of 23 Spanish patients (22 families) with LAL deficiency and the haplotypes associated to the mutations.

We analysed 23 patients (13 WD and 10 CESD) originated from different regions of Spain. The LAL activity was measured using fluorimetric assays. *LIPA* gene was sequenced in genomic DNA using Sanger methodology.

In our series of patients, we identified eight different mutations, four of them not previously reported. The novel mutations were p.H86Y (c.256C>T), p.D352del (c.1055_1057delACG), c.966+2T>G and a change in the same base as the common mutation c.894G>A that causes a splicing defect but also an amino acid change, c.894G>C (p.Q298H). The novel c.966+2T>G mutation accounted for 75% of the WD alleles, and the frequent CESD associated c.894G>A mutation accounted for 55% of the CESD alleles in our cohort. Haplotype analysis showed that both mutations co-segregated with a unique haplotype suggesting a common ancestor.

Our study contributes to the LAL deficiency acknowledgement with novel mutations and with high frequencies of some unknown mutations for WD. Moreover the two common mutations in our cohort are associated to a unique haplotype, suggesting a common ancestor.

P-33

Longitudinal *in vivo* monitoring of disease progression and viral mediated gene injection therapy in ovine Batten disease

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The neuronal ceroid-lipofuscinoses (NCLs; Batten disease) are a group of fatal neurodegenerative lysosomal storage diseases of children caused by a large number of mutations in up to 13 different genes. Forms associated with mutations in two of these, *CLN5* and *CLN6,* are being investigated in well-established sheep models. Defining common features are progressive atrophy of the brain and retina, leading to psychomotor degeneration and blindness. Recent and on-going viral mediated gene therapy trials in the sheep are yielding results encouraging for clinical translation to humans[1]. *In vivo* assessments of brain atrophy and visual impairment are integral to the longitudinal monitoring individual animals and provide robust data for translation to treating human patients.

Blindness in both sheep forms has a central and a peripheral component, as the visual cortex and the retina are both affected, thus it is important to monitor each separately to determine targets for treatment. Observations of the sheep in the field and during maze testing reveal occurrence of visual impairment that correlates with the onset of atrophy of the visual cortex (around 6 months of age), whereas electroretinography (ERG) indicates a later onset and slower development of retinal degeneration. In *CLN5-/-* sheep, the amplitudes of both a- and b-waves in the scotopic ERG become reduced from 7 months compared with controls ($p < 0.05$). *CLN6 -/-* animals show a slightly later onset of retinal degeneration, with both a- and b-wave amplitudes reduced from 11 months ($p < 0.05$). Computed tomography (CT) imaging shows an increase in skull thickness and a progressive reduction of intracranial volume (ICV) in affected animals. This reflects the course of cortical brain atrophy which generalises from the parieto-occipital cortex. ICVs of NCL affected sheep increase for the first few months, as in controls, but then decline progressively between 5 and 13 months in *CLN5 -/-* sheep and 11 to 15 months in *CLN6-/-* sheep ($p < 0.05$). Cerebral ventricular volume also increase with disease progression. These findings have been verified by magnetic resonance imaging (MRI) through which the regionality of brain atrophy can be visualised in greater detail. The combination of these modalities (ERG, CT and MRI) is integral to successful monitoring of large animal treatment trials and to obtain information for human translation.

1. Mitchell NL et al (2017) 21st ESGLD Workshop Abstract

P-34

Model of CNS involvement for mucopolysaccharidosis type II: neural cells from induced pluripotent stem cells of a patient

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As one of X-linked recessive lysosomal storage disorder, mucopolysaccharidosis type II (MPSII, Hunter syndrome) is caused by mutations in the iduronate-2-sulfatase gene (IDS gene, Xq28). IDS defect leads to an accumulation of two main glycosaminoglycans (GAGs) - dermatan sulfate and heparan sulfate all over the body. Symptoms associated to this defect are skeletal deformities, hearing loss, airway obstruction, hepatosplenomegaly, cardiomyopathy and progressive neurological impairment.

Even though studies dealing with CNS pathology of MPS II on mouse models delivered interesting results, they may not accurately reflect the conditions in human. Thus modeling human diseases with induced pluripotent stem cells (iPSC) has remarkable potential to generate new insights into understanding disease pathogenesis and to study possible therapeutic approaches.

We performed reprogramming of patient's blood cells with MPSII into iPSC, and the generated iPSC expressed pluripotency markers and can be potentially differentiated to all three germ layer. We have successfully differentiated iPSC into neurons and glial cells and used them for pathophysiological studies. Particularly, our experiments were focused on jet poorly understood GAG storage pattern in human neural cell types. We have also studied effect of enzyme replacement therapy (ERT) on these cells. Immunofluorescent characterization of the cell types confirmed presence of β-Tubulin III and MAP2 positive neurons, GFAP positive astrocytes and CNPase positive oligodendrocytes. Lysosomal aggregation was detected mainly in glial-iPSC of patient by co-localization of neural markers with lysosomal marker Lamp1 and Cathepsin D. Analysis by electron microscopy showed lysosomal abnormalities in glial-iPSC of patients compared to control. Quantitative determination of GAG by photometric methods revealed moderate increase in GAG level in neural-iPSC with deficient IDS activity.

P-35

Successful desensitization to enzyme replacement therapy by using omalizumab in a patient with late-onset Pompe disease

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Late onset Pompe disease (LOPD) is characterized by progressive motor and respiratory impairment that can lead to severe disability. The specific enzyme replacement therapy (ERT), consisting in intravenous infusions of recombinant alfa glucosidase (rhGAA) every other week, is generally safe and well tolerated, but severe hypersensitivity reactions IgE mediated have been described. Since ERT is fundamental for the long term treatment of LOPD and, at present, no alternatives are available, in these cases a desentization may be essential.

We report a clinical case of a 21 years old man affected by LOPD, who developed a severe allergic reaction during the infusion of rhGAA, after 8 years of regular treatment. The reaction was characterized by urticaria, swelling of the lips and breathing difficulty. Both specific immunoglobulin E (IgE) versus rhGAA and the skin allergy testing with rhGAA resulted positive. Desensitization was tried first with a protocol consisting in delivering solutions with 5 increasing concentrations of rhGAA with slow (6 h) speed rate, which failed to induce immune tolerance. Subsequently, treatment with omalizumab, a monoclonal antibody that specifically binds human IgE, administered subcutaneously, was started.

After two months of treatment, omalizumab was able to counteract the allergic manifestations and normalize the skin allergy test, therefore regular ERT infusions were restarted. After 18 months of combined therapy with omalizumab and ERT, rhGAA-specific IgE became undetectable. Two years later the patient was still free of allergic symptoms, nevertheless he started to complain about severe myalgia. Therefore, we decided to suspend omalizumab and allergic manifestations occurred again after 3 ERT infusions.

To our knowledge, this is the first reported case of a severe allergy to the ERT successfully treated with omalizumab in a patient with LOPD. Concerns about myalgia, as a side effect of the combined therapy ERT+ omalizumab, need further investigations.

P-36

Next generation sequencing strategy for lysosomal storage diseases diagnosis

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The lysosomal storage disorders (LSD) diagnosis may be hampered by their significant heterogeneity with phenotypic overlap, variable severity and onset. Early diagnosis, including genetic testing, may enable treatment in some cases and thus prevent clinical complications. Conventional biological diagnosis procedures are based on a series of sequential investigations and require multiple samples and steps. To overcome these issues, we have developed a Next Generation Sequencing (NGS) panel that includes 51 LSD genes. This panel has been tested in patients with storage features such as bone abnormalities, organomegaly, central nervous system dysfunction and coarse hair and facies or with a non-specific "storage-like" presentation or hydrops fetalis presentation. The design of the lysosomal storage disease panel covered the coding region, promoter region and the flanking intronic sequences for 43 genes. In addition, 3' untranslated sequences were included for 2 genes (AGA and ARSA) and the entire gene sequences were covered for 6 genes (ARSB, CLN3, CLN8, IDS, SGSH and NAGLU). Library construction was done using SureSelect QXT (Agilent) and sequencing was performed on MiSeq instrument (Illumina®). Eighteen patients with LSD with 30 known pathogenic variations and 70 variations using Sanger method were included for validation purposes. The current protocol was designed to detect copy number alterations. The coverage for the targeted exonic regions was excellent (the mean read depth for the designed panel was 492-fold, and 96% of the bases were covered by more than 30 reads.). Expected 100 known variations were found in all patients. NGS combined with robust bioinformatics analyses is a very useful tool for identifying the causative variations of LSD.

P-37

Metabolic causes of non immune hydrops fetalis: next generation sequencing panel as first line investigation

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Background: Hydrops fetalis is a life-threatening fetal state and a subset of this condition is classified as nonimmune hydrops fetalis (NIH) and comprises 90% of all cases. Up to 15% of NIH may be due to inherited metabolic disorders. But a large part of NIH linked to metabolic disorders remains undiagnosed due to partial and sequential investigations. This approach is time consuming and the success in identifying a cause is limited. Therefore, we have developed a Next Generation Sequencing (NGS) panel to investigate metabolic causes of NIH. Among the inherited metabolic diseases underling NIH, the most severe forms of lysosomal storage diseases (LSD) represent a large part. At least 15 LSD are linked to NIH. Besides, 26 other inherited metabolic diseases have been described in this condition (Glycogenosis, Peroxisomal disorder, Long-chain-hydroxyacyl CoA dehydrogenase deficiency, Citric-acid cycle defect, Transaldolase deficiency).

Method: The design of the hydrops fetalis (HydFet) panel covered the coding region, promoter region and the flanking intronic sequences for 41 genes. Library construction was done using SureSelectQXT (Agilent) and sequencing was performed on MiSeq instrument (Illumina®). Thirty known pathogenic variants and 70 benign variants using Sanger method were included for the method validation.

Results: This method allows the analysis of greater than 82.1 of the targeted sequence. The current protocol was designed to detect copy number alterations. The data were obtained with an excellent coverage for all targeted regions (92.5% of the targeted sequences were covered more than 30-fold and the average coverage was 408-fold). Expected 100 known variations were found in all patients. Sixty NIH samples have been analyzed using this method.

Conclusion: NGS combined with robust bioinformatics analyses is a very useful tool for identifying the causative variants of NIH. Additional biochemical investigations of the protein encoded by the altered gene may confirm the diagnosis. This paradigm shift may allow enhancing the NIH diagnosis rate.

P-38

Mucopolysaccharidosis Type VI (MPS VI) and Molecular Analysis: A Review of Published Classified Variants in the *ARSB* Gene

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MPS VI (Maroteaux-Lamy syndrome) is an autosomal recessive lysosomal storage disorder caused by mutations in the ARSB gene that result in deficient activity of the lysosomal catabolic enzyme N-acetylgalactosamine-4-sulfatase (arylsulfatase B, ASB). Deficient ASB activity causes lysosomal storage and elevated urinary excretion of the glycosaminoglycan enzyme substrates dermatan sulfate and chondroitin-4-sulfate. MPS VI is diagnosed through clinical findings and deficient ASB enzyme activity with normal activity of control enzymes; detection of pathogenic variants in each ARSB allele can independently confirm the diagnosis and facilitate genetic counseling. A previous report (Karageorgos et al. 2007) on a cohort of 105 individuals diagnosed with MPS VI documented that most ARSB variants are rare or private. Since 2007, with the increasing use of molecular testing, many new ARSB variants have been published. To uniformly summarize all ARSB variants, we collected and analyzed from the literature and public databases 822 reports of 193 distinct variants in the ARSB gene from individuals diagnosed with MPS VI. In agreement with previous reports (Karageorgos et al. 2007), most variants are missense (60%; 115 of 193); next most common are deletions (18%; 34 of 193), followed by nonsense (12%; 23 of 193), and splice site/intronic variants (6%; 11 of 193). Many reported ARSB variants are rare, with 33% (64 of 193) reported only once. Zygosity of individuals with MPS VI (n=411) distributed as: 51% homozygous (209 of 411) and 42% heterozygous (173 of 411); in 7% of cases only one allele was identified (29 of 411). Of the 193 unique ARSB variants summarized here, only 18% (34 of 193) are recorded in public databases in association with supporting evidence/clinical significance. This analysis illustrates the heterogeneity of alleles linked to MPS VI and lack of representation of otherwise characterized pathogenic ARSB alleles in publicly available variant databases. We emphasize the importance of maintaining high clinical suspicion during MPS VI diagnosis and confirming diagnosis via ASB enzyme testing as many ARSB alleles may have yet to be formally classified as MPS VI-associated. Timely submission and classification of ARSB variants in public databases in association with biochemical and clinical data will help improve a timely diagnosis of MPS VI.

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Antisense based correction of *GAA* splicing in iPSC-derived skeletal muscle cells from Pompe patients that carry the IVS1 variant

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The most common variant causing Pompe disease is c.-32-13T>G (IVS1) in the acid α-glucosidase (*GAA*) gene, which weakens the splice acceptor of *GAA* exon 2 and induces partial and complete exon 2 skipping. It also allows a low level of leaky wild type splicing, leading to a childhood/adult onset phenotype. We hypothesized that cis-acting splicing motifs may exist that could be blocked using antisense oligonucleotides (AONs) to promote exon inclusion. To test this, a screen was performed in patient-derived primary fibroblasts using a tiling array of *U7* snRNA-based AONs. This resulted in the identification of a splicing regulatory element in *GAA* intron 1. We designed phosphorodiamidate morpholino oligomer-based AONs to this element, and these promoted exon 2 inclusion and enhanced GAA enzyme activity to levels above the disease threshold. To test how these AONs would affect *GAA* splicing in skeletal muscle cells, we expanded iPSC-derived myogenic progenitors and differentiated these to multinucleated myotubes. AONs restored splicing in myotubes to a similar extent as in fibroblasts, suggesting that they act by modulating the action of shared splicing regulators. AONs targeted the putative polypyrimidine tract of a cryptic splice acceptor site that was part of a pseudo exon in *GAA* intron 1. Blocking of the cryptic splice donor of the pseudo exon with AONs likewise promoted *GAA* exon 2 inclusion. The simultaneous blocking of the cryptic acceptor and cryptic donor sites restored the majority of canonical splicing and alleviated GAA enzyme deficiency. These results highlight the relevance of cryptic splicing in Pompe disease, and its potential as therapeutic target for splicing modulation using AONs.

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Lysosomal dysfunction in Smith-Lemli-Opitz syndrome caused by inhibition of the NPC1 protein can be corrected using some NPC therapies.

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Smith-Lemli-Opitz syndrome (SLOS) is a rare developmental disorder caused by mutations in 7-dehydrocholesterol reductase, which converts 7-dehydrocholesterol to cholesterol in the final step of the cholesterol biosynthetic pathway. Children with SLOS are characterised by autistic spectrum disorders, mental retardation, and birth defects including cleft palate and syndactaly of the second and third toes. There is a wide range of severity, from prenatal loss to near-normality. There is currently no effective therapy – increasing dietary cholesterol is of no benefit due to impaired cellular endocytic trafficking.

In culture, SLOS cells do not show any abnormalities, as cell culture medium is rich in cholesterol, meaning the cells to not rely on the biosynthetic pathway so do not produce 7-dehydrocholesterol. However, when grown in lipoprotein deficient medium, which contains very little cholesterol, cells are characterised by elevated 7-dehydrocholesterol, impaired endocytic trafficking, increased lysosomal volume, and intralysosomal storage of cholesterol (when added back to the culture medium), sphingomyelin, and glycosphingolipids. This combination of phenotypes is characteristic of the lysosomal storage disorder Niemann-Pick type C (NPC). Pharmacological inhibition of 7-dehydrocholesterol reductase in zebrafish causes developmental abnormalities, reduced spontaneous movement and a slow heartbeat. We have found that 7-dehydrocholesterol, which accumulates in SLOS cells grown in lipoprotein deficient serum, inhibits the NPC1 protein and causes the NPC-like phenotypes. We therefore tested NPC therapies in SLOS cells and in a zebrafish model to determine whether these were of benefit in SLOS.

We found that treatment of SLOS cells or zebrafish with miglustat improved all measureable SLOS phenotypes. However, treatment with experimental NPC therapies cyclodextrin and curcumin did not improve phenotypes in SLOS cells. Curcumin mediates benefit in NPC by releasing Ca^{2+} from the endoplasmic reticulum, which compensates for defective lysosomal Ca^{2+} signalling in NPC and improves endocytic trafficking. Curcumin does not release Ca^{2+} in SLOS cells. This explains the lack of efficacy and uncovers an important new Ca^{2+} signalling defect in SLOS. In conclusion, we have identified the mechanism behind lysosomal dysfunction in SLOS and can rescue this using miglustat, and have uncovered a novel Ca^{2+} signalling defect in SLOS.

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A targeted sequencing panel for the analysis of exons and conserved intronic sequences of 50 LSD genes

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The diagnosis of Lysosomal Storage Disorders (LSD) could be very difficult and may take several years, given also the shared clinical features of many LSDs. In this study we evaluated an LSD targeted sequencing panel as a diagnostic tool. We used the Ion AmpliSeq platform (Thermo Fisher Scientific®) for the design of a custom panel including 50 LSD genes. For each gene, all protein-coding transcripts, including exons, exon flanking sequences (50 bp on each side) and both UTRs were considered in the panel design. We also included 230 Conserved Intronic Fragments (CIF) obtained comparing the intronic sequences of 33 placental mammals. The panel design output was of 187.42 kb, covered by 1561 amplicons representing 93% of the target sequence. As a validation, we analyzed 80 patients including 59 positive controls (PC), 12 biochemically diagnosed patients (BD) and 9 undiagnosed patients (UD). Library preparation was performed using the Ion AmpliSeq™ DNA Library Preparation kit (Thermo Fisher Scientific®). For the variants analysis we used the QueryOR platform (http://queryor.cribi.unipd.it), setting the coverage for each allele >10X and the allele frequency <0.01. All identified variants were verified through IGV (Integrative Genomics Viewer) and annotated on the basis of the HGVS nomenclature through Ensembl VEP (Variant Effect Predictor).

The analysis lead to the identification of pathogenic variants in 64% of the PC alleles and in 61% of BD alleles. This may be due to the presence of uncovered/poorly covered regions (excluded from the panel design or scarcely amplified in the enrichment phase) and to the presence of large deletions. Two out of 9 undiagnosed patients were diagnosed.

Six UD patients and three BD patients were further analyzed by Whole Exosome Sequencing (WES), by using the Illumina platform. Exome analysis of variants is still ongoing; up to date WES was resolving for one UD and one BD patients.
Although with some limitations, targeted sequencing is an appealing approach to implement routine diagnostic strategy, given its low sequencing costs and short sequencing time.

Acknowledgements: this work was supported by the University of Padua, Strategic Project BIOINFOGEN

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Developing substrate reduction therapy for Metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD) is caused by the deficiency of arysulfatase A. This leads to the accumulation of sulfo-galactosylceramide (sulfatide) which results in progressive demyelination in the patients. One of the therapeutic options which may be applicable to treat MLD is substrate reduction therapy. The target of this therapy is cerebroside-sulfotransferase (CST). This enzyme transfers a sulfonic acid residue from PAPS to galactosylceramide which yields sulfatide. CST is a membrane bound Golgi located enzyme. Thus, for substrate reduction therapy an inhibitor for CST must be identified. We developed a robust fluorescence based assay to follow CST activity. This assay is based on an engineered CST which is soluble, secreted and can be easily purified by affinity chromatography. The assay is linear over time and different protein concentrations. We applied this assay to screen two libraries (total of ~ 75.000 compounds) based mostly on synthesized compounds. No hit was identified in these screens. We then switched to natural compound libraries. In one screen we identified Epicoccolid, a secondary metabolite purified from the marine fungus Epicoccus nigra which inhibits CST by 50% at a concentration of 1 µM. We extended our high throughput screening to a library based on ~200.000 fractions derived from a variety of natural sources. After several rounds of hit confirmation using the classical CST assay based on the use of radioactive PAPS finally 28 fractions were confirmed to contain natural compounds inhibiting CST. Among these 28 fractions four were identified which in dilutions of at least 1:100 still inhibited CST by more than 50%. We believe that these fractions provide a good basis to identify therapeutically applicable inhibitors of CST.

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