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Research Project

In vitro studies of energy dysfunction in classical-infantile and late-infantile Neuronal Ceroid Lipofuscinosis

Annual report

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IN VITRO STUDIES OF ENERGY DYSFUNCTION IN CLASSICAL-INFANTILE AND LATE-INFANTILE NEURONAL CEROID LIPOFUSCINOSIS

BACKGROUND

The neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease, are a heterogeneous group of progressive neurodegenerative disorders affecting children and adults, characterized by retinopathy leading to blindness, ataxia and gait abnormalities, drug-resistant progressive myoclonic epilepsy, mental deterioration, and an early death. The NCLs represent the most common childhood lysosomal storage disorders (LSDs) and show a characteristic accumulation of autofluorescent ceroid lipopigments. These abnormal cytosomes are made of, subunit c of mitochondrial ATP synthase or sphingolipid activator proteins A and D and are localized in lysosomes of most cells, including neurons, skin fibroblasts and blood lymphocytes.

The genetic landscape of NCL is highly heterogeneous and mutations in 13 different genes (*CLN*s) underlie various subtypes, yet about 8% of the cases remain undefined. In patients manifesting clinical features in infancy mutations in the *PPT1*/CLN1 gene — encoding palmitoyl-protein thioesterase-1 (PPT1) — cause infantile NCL (INCL) are relatively frequent whereas mutations in *CTSD*/CLN10, coding for cathepsin D (CTSD), underlying the congenital-onset NCL (CNCL) are less common.

Cathepsin D (CTSD) is a major lysosomal aspartic protease in lysosomes. Lysosomal CTSD activity catalyzes degradation and clearance of exogenous as well as endogenous macromolecules and damaged organelles delivered to the lysosomes. Intracellular accumulation of undigested long lived proteins and other macromolecules underlies the pathogenesis of several neurodegenerative disorders.
The synthesis of CTSD occurs in the endoplasmic reticulum (ER) as a pre-pro-peptide with a molecular mass of ~50 KDa. The cleavage of the leader peptide in the ER generates the 48 kDa form, precursor of mature-CTSD (CTSDpro). In the Golgi complex, attachment of mannose 6-phosphate (M6P) moieties to CTSDpro facilitates its binding to endosomal/lysosomal sorting receptors. The receptor-ligand complexes then exit the trans-Golgi network (TGN) in clathrin-coated intermediates and fuses with the endosomal system. The low pH of the late endosomal lumen facilitates dissociation of the receptor-ligand complexes and allows the ligand (i.e. pro-CTSD) to be delivered to lysosome. The CTSDpro then undergoes further proteolytic cleavage by cathepsin B and cathepsin L, which generates the 31 kDa and 14 kDa fragments, respectively, and their non-covalent dimerization constitutes the mature-, catalytically active-CTSD (CTSDm).

CLN1 gene encodes the lysosomal enzyme palmitoyl protein thioesterase 1 (PPT1) that cleaves the thioester linkage in S-acylated proteins (constituents of ceroid) required for their degradation by lysosomal hydrolases. The exact physiological function of PPT1 remains yet unclear, though it is proposed that PPT1 participates in endocytosis, vesicular trafficking, synaptic function, and cell death signaling.

Mutations in *PPT1/*CLN1 are associated with ultrastuctural evidence of granular appearance of stored material (granular osmiophilic deposits, GRODs) which represent the hallmark of proteolipidic storage common in CLN1 disease.

The understanding of the cellular mechanisms involved in neuronal injury in NCLs, as well as the function of most affected proteins remains largely elusive. Recent works support the hypothesis of interactions between NCL proteins occurring at multiple points along a single pathway. In this regard a common pathogenic link between INCL and CNCL has been proposed, suggesting that defect in *Cln1*$^{-/-}$ mice lead to a defective processing of pro- to mature-CTSD in lysosome with ensuing CTSD deficiency and the correlated neuropathology.

OBJECTIVES

The overall aim of this research program during the the PhD course is to investigate the pathogenetic mechanisms underlying different forms of NCL in order to study common molecular signatures for the identification of biomarkers and modifying factors involved in the disease.

During this first year of my studies I had the opportunity to perform the genetic and biochemical characterization of a novel *CLN10* mutation in relation with common pathways implicate in the disease mechanisms such as autophagy, lysosomal alterations and ultrastructural abnormalities. The possibility that defect in CTSD maturation process lead to alterations in PPT1 mRNA expression levels was also evaluated

RESULTS AND DISCUSSION

We studied a 2-year-old girl who presented psychomotor regression and drug-resistant epilepsy. We initially ruled out mutations in *CLN1/PTT1* gene.

On the other hand, molecular testing of the *CLN10* gene identified a novel homozygous variation (c.205G>A/p.Glu69Lys) in exon 2. The mutation was homozygous in the patient, heterozygous in healthy parents and absent in large exome polymorphic databases (ExAC, ESP6500). *In silico* predictions suggested a probably damaging role of a highly conserved amino acid.

We then investigated the expression levels of CTSD in cultured skin fibroblasts obtained from the patient by western blotting analysis. We detected low levels of CTSDm and even though the pro and the single chain forms of CTSD were barely visible, they seemed to be normally expressed suggesting a not complete process of protein maturation, most likely due to the mutation (Figure 1).

To evaluate the effects of the novel mutation on CTSD enzyme activity, we established a collaboration with prof. Michael Przybylsk (Biomedical Mass Spectrometry Unit, University of of Konstanz, Germany) to set up in a dried blood spots (DBS) the activities of NCL-related lysosomal hydrolases (the enzyme activities of CLN1, CLN2, CLN10 and CLN13) in patients through tandem mass spectrometry. We also evaluated DBS from healthy carriers and age/sex-matched control individuals. The concentration of the reaction product after a defined time were then translated into enzymatic activity in μM/L blood/h using an internal standard as reference. The enzymatic results clearly showed a reduced activity in the child harboring the novel p.Glu69Lys in *CTSD* carrier parents showed values intermediate between controls and the patient (Figure

2).The activities of CLN1/PPT1 and CLN2/TPP1 in the same samples were normal in mass spectrometry (not shown). These results corroborated the pathogenic significance of the novel mutation in *CTSD* and suggested a loss of function mechanism.

The electron microscopy of patient' skin biopsy showed evidences of granular appearance of stored material (granular osmiophilic deposits, GRODs) (Figure 3A) with the same ultrastructural characteristics seen in CLN1 disease.

To test the possible effects of the novel mutation in *CTSD* on PPT1 mRNA expression, we analyzed by qPCR following standards protocols mRNA expression in cultured skin fibrobalsts and did not observe significant modifications (Figure 3B); suggesting lack of correlation with the accumulation of stored material and that the ultrastructural GRODs abnormalities observed are unlikely related to a downregulation of PPT1 activity but rather close linked to the novel mutation in *CTSD*.

It is known that mature cathepsin D is involved in autophagy and plays a crucial role in the control of cell and tissue homeostasis. In our previous work in cells from patients harboring mutations in *CTSF*/CLN13 (DiFabio et al. 2014) we demonstrated a role of cathepsin F, a cysteine protease part of the same family of cathepsin D, in the production of aggresome-like inclusions inducing autophagic pathway in a family with Kufs disease type B (an adult form of NCL). With this background, we performed a similar study of the autophagic process in cultured skin fibroblasts of the new *CTSD*/CLN10 patient presenting the clinical phenotype of CNCL.

We observed that the aggresome formation using an aggresome detection dye (red fluorescence), which is essentially non-fluorescent until it binds to the structural features associated with aggregated protein cargos. unlike CTSF fibroblasts showing cytoplasmic inclusions suggestive of lipofucsin accumulation, there were no aggresome-like inclusions accumulating in CTSD patient's fibroblasts (Figure 4).

By western blotting analysis we also evaluated the expression of LC-III and p62, the major biomarkers of autophagy, in basal condition and after appropriate stimuli with torin1 (an activator of autophagy through the inhibition of mTOR pathway) and cloroquine (an autophagy inhibitor through lysosome neutralization and inactivation of lysosome enzymes) (Figure 5A). The p62/SQSTM1 protein is a ubiquitin-binding scaffold protein that localizes with ubiquitinated protein aggregates in many neurodegenerative diseases. p62/SQSTM1 binds directly to LC-III family proteins via a specific sequence motif. The protein is itself degraded by autophagy and may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome. Since p62 accumulation occurs when autophagy is inhibited, and decreased levels can be observed when autophagy is induced, p62 may be used as a marker to study the accuracy of the autophagic flux.

By fluorescent microscopy we labeled lysosomes in patient's live cells (Figure 5B) evaluating the lysosomal compartment to observe the number and distribution of lysosomal pattern that might result dysregulated if the autophagic pathway is disrupted.

Our results suggest a correlation between the data on aggresome formation and the biomarkers of autophagy: a normal pattern of autophagy process and flux is achieved in CTSD patient's fibroblasts and, at least in peripheral cells from this case, it does not seem to indicate a disrupted autophagy role in the disease

processes. This is different from what we had observed in peripheral cells from patients harboring mutations in the CLN13 gene, a different form of NCL associated with another cathepsin (i.e., CTSF).

FUTURE DIRECTIONS

In the near future, we will attempt to clarify the role of human CLN5 in a neuronal cell models, analyzing proteome and interactome expression profiles, in collaboration with researchers at the Meilahti Clinical Proteomics Core Facility, Institute of Biomedicine/Biochemistry and Developmental Biology, University of Helsinki. Neuronal cell model of CLN5 harboring three different CLN5 Italian mutations will be developed through site-direct gene editing and analyzed in parallel with brain tissues from *Cln5*$^{-/-}$ KO mice. This further step of the research project will be relevant to outline the function of CLN5p within the intricate NCL molecular network.

REFERENCES

Cathepsin D: a cellular roadmap.
Zaidi N, Maurer A, Nieke S, Kalbacher H. Biochem Biophys Res Commun. 2008

Cln1 gene disruption in mice reveals a common pathogenic link between two of the most lethal childhood neurodegenerative lysosomal storage disorders.
Chandra G, Bagh MB, Peng S, Saha A, Sarkar C, Moralle M, Zhang Z, Mukherjee AB. Hum Mol Genet. 2015

Genetic basis and phenotypic correlations of the neuronal ceroid lipofusinoses.
Warrier V, Vieira M, Mole SE. Biochim Biophys Acta. 2013

Human pathology in NCL.
Anderson GW, Goebel HH, Simonati A. Biochim Biophys Acta. 2013

Molecular epidemiology of childhood neuronal ceroid-lipofuscinosis in Italy.
Santorelli FM, Garavaglia B, Cardona F, Nardocci N, Bernardina BD, Sartori S, Suppiej A, Bertini E, Claps D, Battini R, Biancheri R, Filocamo M, Pezzini F, Simonati A. Orphanet J Rare Dis. 2013

Neuronal ceroid lipofuscinosis: a common pathway?
Persaud-Sawin DA, Mousallem T, Wang C, Zucker A, Kominami E, Boustany RM. Pediatr Res. 2007

Pseudo-dominant inheritance of a novel CTSF mutation associated with type B Kufs disease.
Di Fabio R, Moro F, Pestillo L, Meschini MC, Pezzini F, Doccini S, Casali C, Pierelli F, Simonati A, Santorelli FM. Neurology. 2014

FIGURES

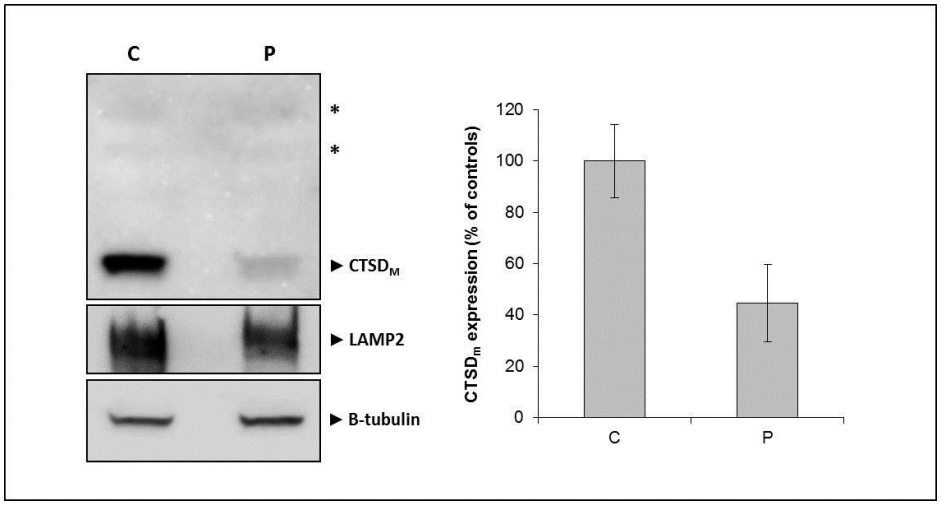

Fig.1 Western blotting analysis of CTSD enzyme in skin fibroblasts from a patient with the novel homozygous G205A CTSD gene mutation (p.Glu69Lys).
The band at 34 kDa representing the mature CTSD. The pro (52 kDa) and the single chain (43 kDa) forms of CTSD (indicated by asterisks) are slightly visible in human fibroblasts. LAMP2 protein has been used as lysosomal marker. β-tubulin staining of the samples shown as a loading control.
Histogram quantify the reduction levels of mature CTSD. Data refers to three independent experiments

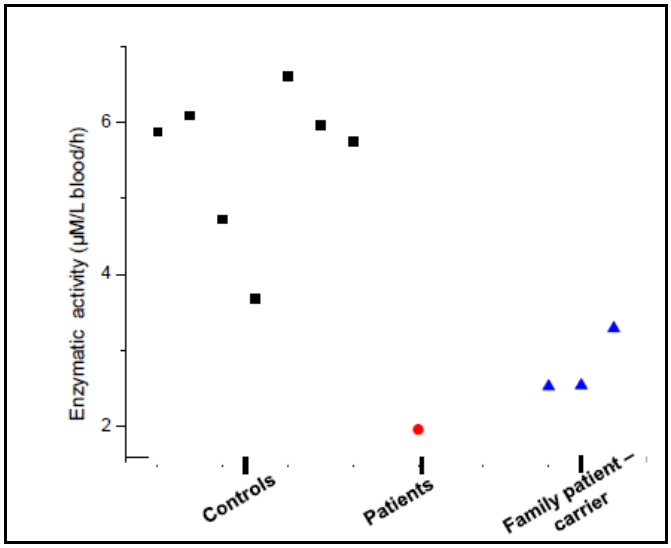

Fig.2 Biochemical analysis of CTSD in DBS obtained from the proposita, her carrier parents and six healthy, age/sex-matched controls. The enzymatic ratio were expressed in µM/L blood/h.

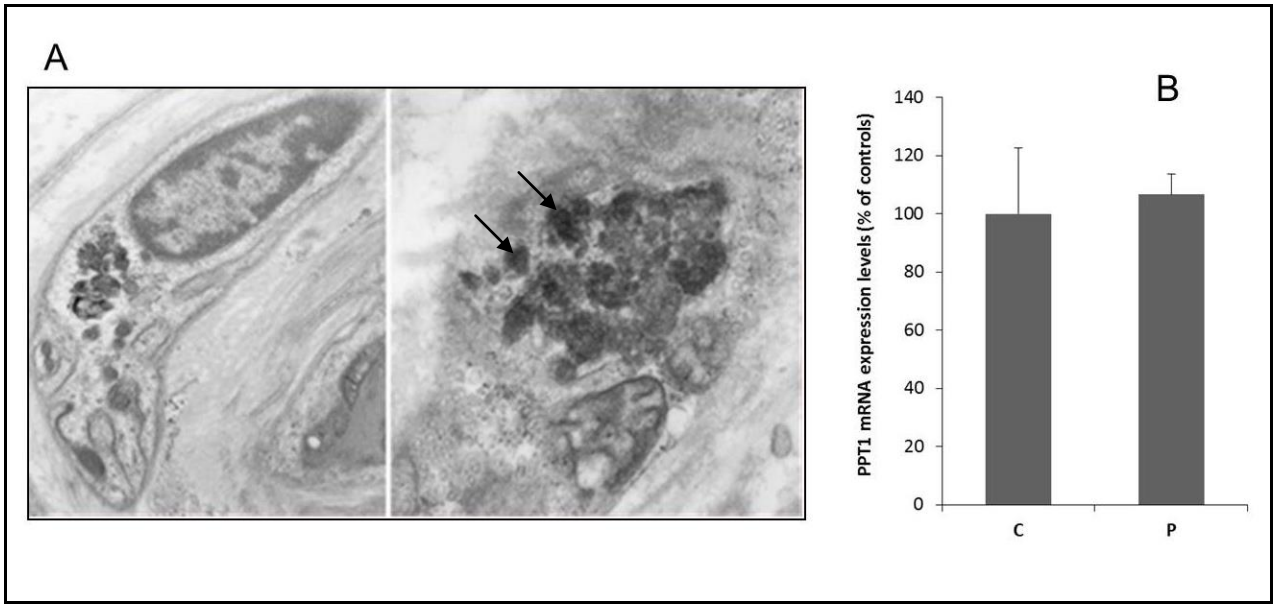

Fig 3. **A** electron microscopy of patient's skin biopsy harboring the homozygous c.205G>A/p.Glu69Lys mutation in *CTSD.*
Arrows point out the granular appearance of stored material (granular osmiophilic deposits, GRODs).
B Analysis of *PPT1*/mRNA expression levels by qPCR.

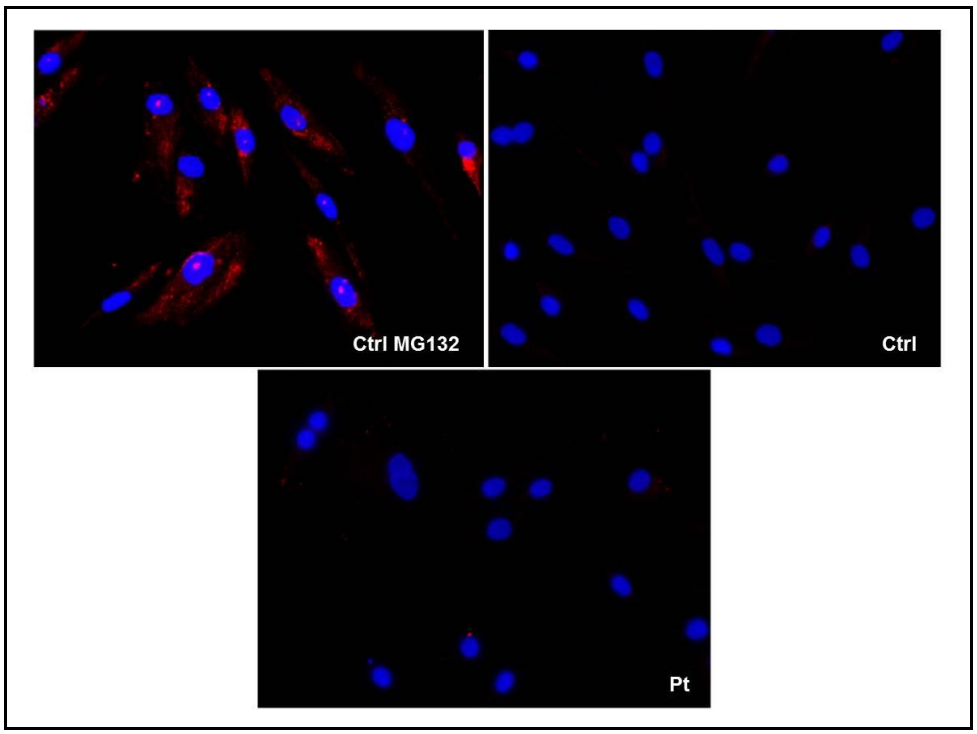

Fig 4. Analysis of aggresome formation.
ProteoStat® aggresome detection dye (red fluorescence) releases fluorescence only when it binds structural features associated with aggregated protein cargos. Hoechst 33342 (blue fluorescence) was used as nuclear stain.. As shown in the picture, no aggresome-like inclusions were found in patient's fibroblasts and in control cells unlike what happens when aggregation was induced in control cells by treatment with the proteasome inhibitor MG-132 in control cells.

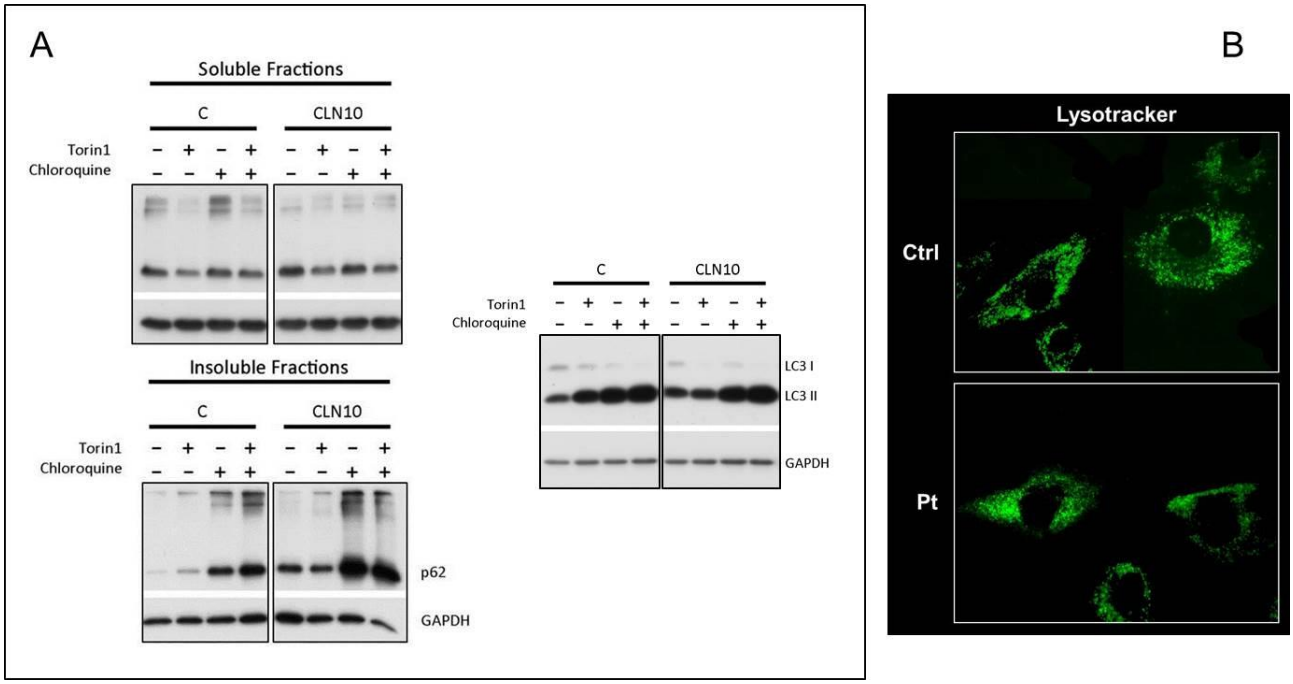

Fig 5. Analysis of autophagy process. **A** Western blotting analysis of the major biomarkers of autophagy in basal condition and after appropriate stimuli.
B Analysis of lysosomal pattern by LysoTracker fluorescent probe, a fluorescent acidotropic marker for labeling and tracking acidic organelles in live cells

OTHER WORKS

During the first year of my PhD program. I also had the opportunity to collaborate in research projects aimed to study different neurodegenerative diseases. These activities produced the following publications:

Powerhouse failure and oxidative damage in autosomal recessive spastic ataxia of Charlevoix-Saguenay
Criscuolo C, Procaccini C, Meschini MC, Cianflone A, Carbone R, **Doccini S**, Devos D, Nesti C, Vuillaume I,Filla A, De Michele G, Matarese G, Santorelli FM.
J Neurol. 2015 *in press*

Mitochondrial respiratory chain defects in skin fibroblasts from patients with Dravet syndrome.
Doccini S, Meschini MC, Mei D, Guerrini R, Sicca F, Santorelli FM.
Neurol Sci. 2015

Additive effect of nuclear and mitochondrial mutations in a patient with mitochondrial encephalomyopathy.
Nesti C, Meschini MC, Meunier B, Sacchini M, **Doccini S**, Romano A, Petrillo S, Pezzini I, Seddiki N, Rubegni A, Piemonte F, Donati MA, Brasseur G, Santorelli FM.
Hum Mol Genet. 2015

Pseudo-dominant inheritance of a novel CTSF mutation associated with type B Kufs disease.
Di Fabio R, Moro F, Pestillo L, Meschini MC, Pezzini F, **Doccini S**, Casali C, Pierelli F, Simonati A, Santorelli FM.
Neurology. 2014