Generation and characterization of a *GALC* knock-out human oligodendrocyte cell line (MO3.13) using CRISPR/Cas9 methodology for studying Krabbe disease.

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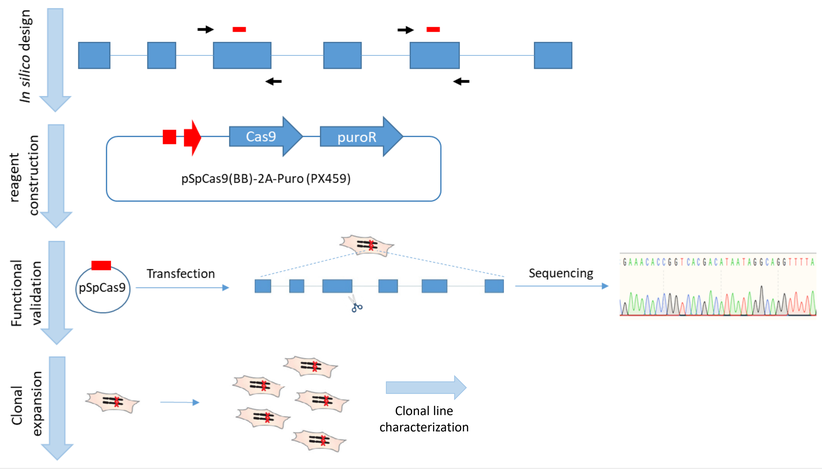
1. BACKGROUND

Krabbe disease (KD) is an inherited lysosomal storage disorder caused by mutations in the *GALC* gene that result in reduced activity of the lysosomal hydrolase, galactosylceramidase (GALC). In the nervous system, GALC is responsible for the catabolism of several galactosphingolipids, such as galactosylceramide (GalCer) and galactosylsphingosine (PSY), playing a critical role in the synthesis and maintenance of myelin. In the absence of GALC, a dysregulated accumulation of PSY alters cellular homeostasis and induces widespread demyelination followed by devasting neurodegeneration. The myelin-forming cells -oligodendrocytes and Schwann cells- are particularly sensitive to GALC dysfunction [1].

	Disease classification	Symptoms	Disease Progression	Median Survival
Early onset	Early infantile 0-6 months	Crying/Irritability, feeding difficulties, poor head control, fisted hands, developmental delay	Progressive neurologic deterioration, seizures, psychomotor regression, loss of vision, hearing and voluntary movement	1.5 - 2 years
Early onset	Late infantile 7-36 months	Similar to early infantile	Similar to early infantile but slower progression	9.5 years
Late onset	Juvenile onset 3-15 years	Vision problems, muscle weakness, gait changes, loss of developmental milestones	Variable progression with older patients generally experiencing slower progression	>16 years
Late onset	Adult onset Above 15 years	Gait disturbance, weakness, lower limb hypoesthesia, cognitive regression, spastic paraparesis	Variable progression with older patients generally experiencing slower progression	>33 years

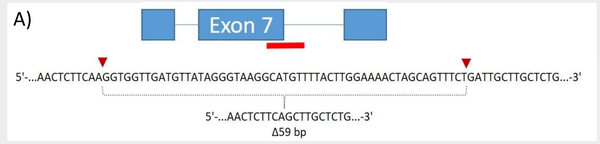
This study aims to generate a stable and easily accessible in vitro model for investigating the effects of GALC deficiency in oligodendrocytes.
This tool might be useful for discovering new druggable targets and testing therapeutic approaches.

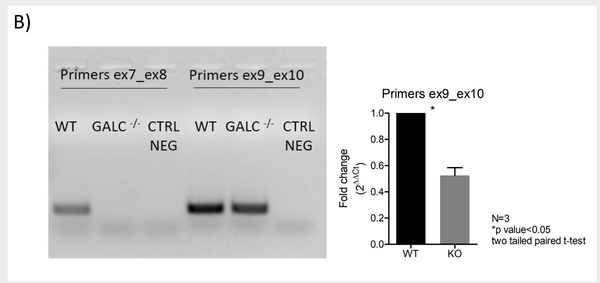
2. METHODS



We exploited the CRISPR/Cas9 methodology to promote genome editing in the *GALC* gene of the MO3.13 cell line [2]. Four gRNAs were designed to target different *GALC* regions (H2, H3, H5, H38). All gRNA sequences were successfully cloned into an expression plasmid bearing gRNA scaffold backbone (BB) (red arrow), Cas9 gene and puromycin resistance gene (puroR), pSpCas9(BB)-2A-Puro (PX459). Sequence-verified plasmids are transfected into human MO3.13 cells and assayed for their ability to mediate targeted cleavage by sequencing. Transfected cells are clonally expanded to obtain an isogenic cell line with a specific mutation.

3. RESULTS





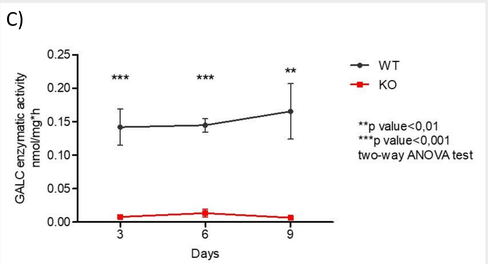
3.1 Generation of stable *GALC*-KO MO313 cells carrying a homozygous deletion in the *GALC* gene. This mutation affects the sequence encoding the nucleophilic domain of the GALC protein.

A) By Sanger sequencing, we identified an isogenic cell population carrying a deletion of 59 nucleotides, 19 in the exon 7 and 40 in the intron 7-8 (GALC $^{-/-}$).

B) We verified GALC deficiency at the transcriptional level by performing Reverse Transcription (RT) PCR, and quantitative RT-PCR.

C) The isogenic GALC $^{-/-}$ MO313 cells show significantly reduced enzymatic activity at different time points of cell seeding (72h, 6 days, 9 days).

HMU-βGal assay was performed, as previously described in Del Grosso et al., 2021 [3].

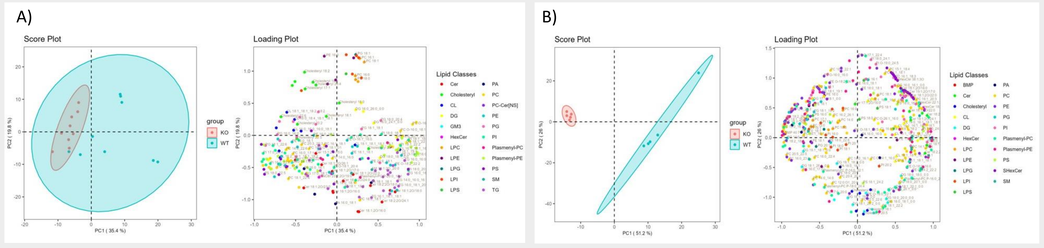


3.2 Lipidomic study of GALC $^{-/-}$ MO313 cells compared to WT ones.

We analysed the lipid profiles of MO313 cells cultured for 3 days in a standard growth medium (SM) and in a differentiation medium (DM) [4] using Mass Spectrometry/Liquid Chromatography (LC/MS) [5].

A) We observed no significant differences in lipid classes in undifferentiated KO cells compared to WT ones.

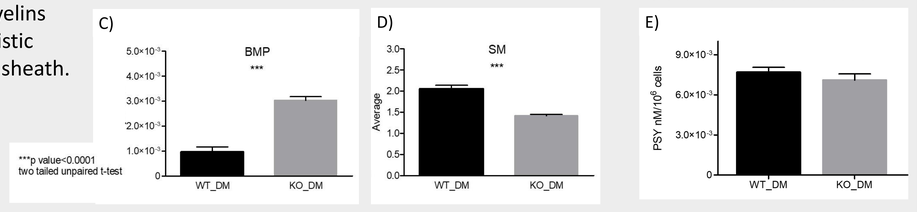
B) Under differentiation conditions, we observed two clusters of comparison between KO and WT cells.



C) BMP is an important class of regulatory lipids markedly enriched in endosomal and lysosomal vesicles.

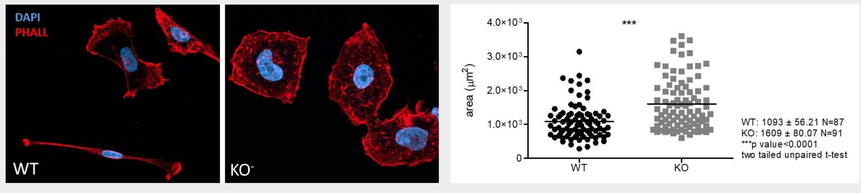
D) Sphingomyelins are characteristic of the myelin sheath.

E) Psychosine (PSY) is a cytotoxic lipid, the most relevant in Krabbe disease.



3.3 Analysis of cell morphology.

We started to explore the effects due to GALC abrogation by measuring the cell spreading area by direct tracing of the cell perimeter, as previously reported in Yu H. et al., 2013 [6].



4. CONCLUSIONS

- Establishment of an isogenic *GALC*-KO cell line with abolished GALC activity.
- Alterations in lipid profile of the differentiated *GALC*-KO cells compared to WT ones.
- Increased BMP levels could be linked to endosomal/lysosomal function.
- Decreased SM levels suggest pathological modification in cellular membranes of KO cells.
- Culture time in the differentiation medium could be little to induce PSY accumulation.
- Preliminary analysis suggest morphological changes in the *GALC*-KO cell line.

5. FUTURE PERSPECTIVES

- In-depth examination of the lipidomic data focusing on specific molecular species and lipid building blocks.
- Optimize the differentiation protocol and analyse PSY dosage.
- Quantitative morphological characterization and the study of focal adhesion dynamics.
- Evaluation of autophagy and apoptosis markers.
- Set up a treatment protocol to rescue GALC activity in the KO cell line.

REFERENCES

[1] Nasir G et al., Front Med. 2021 Nov 11;8:760236.
[2] Giuliano CJ et al., Curr Protoc Mol Biol. 2019 Sep;128(1):e100.
[3] Del Grosso A et al., JIMD Rep. 2021 Nov 12;63(1):50-65.
[4] Voccoli V et al., Cell Death Dis. 2014 Nov 20;5(11):e1529.
[5] Del Grosso A et al., Neurobiol Dis. 2019 Sep;129:195-207.
[6] Yu H et al., Adv Healthc Mater. 2013 Sep;2(9):1188-97.