SAPIENZA
UNIVERSITÀ DI ROMA

Metachromatic Leukodystrophy (MLD) Gene Therapy on ARSA gene via CRISPR/Cas9

Postgraduate Master

"Stem cell and genome editing (U-STEM) In memoriam of Paolo Bianco"

Academic Year 2018/2019

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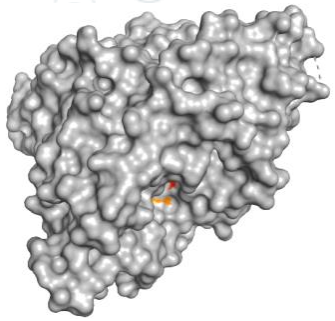
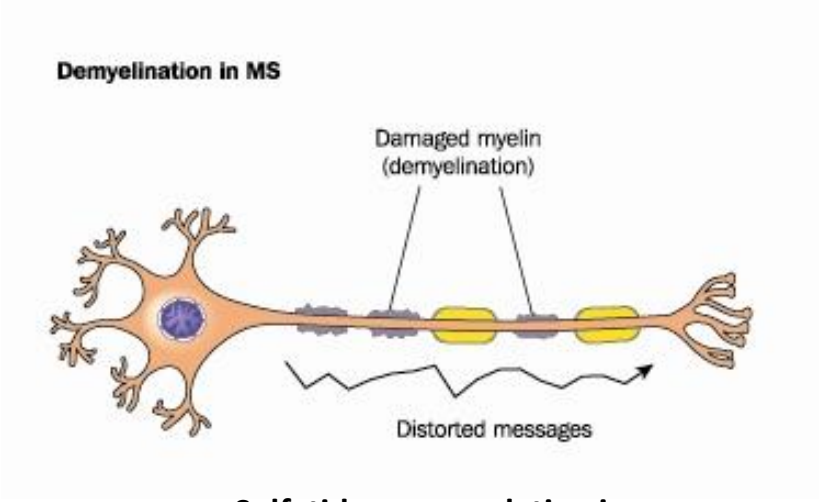
MLD: BACKGROUND

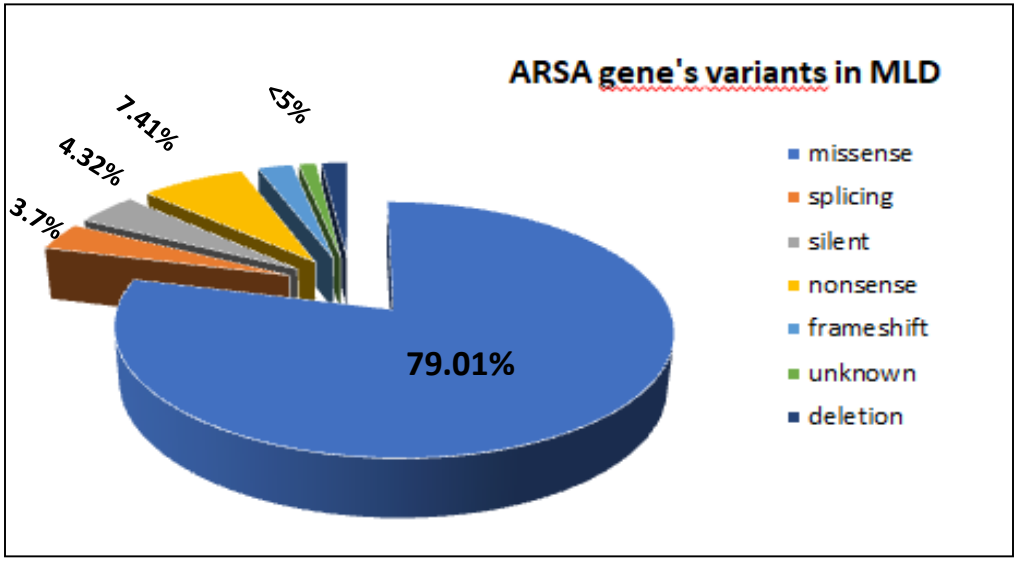


ArylSulfatase A enzyme (ARSA)

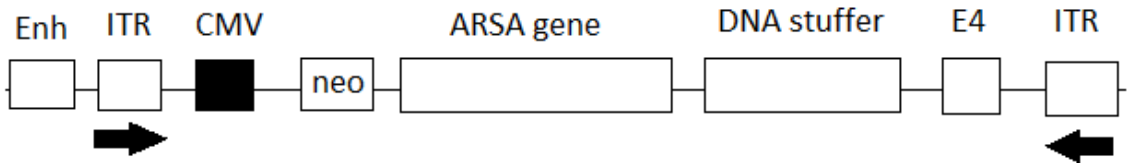
Sulfatides Galactocerebrosides



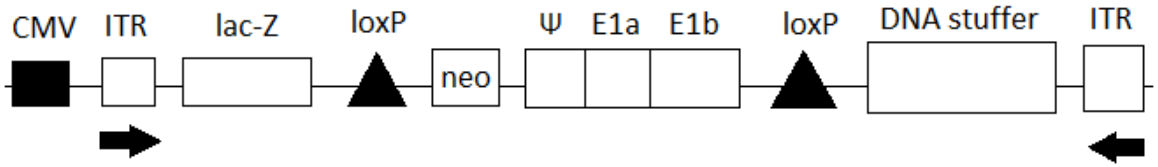
Sulfatides accumulation in oligodendrocytes and Schwann cells



AIM AND APPROACH

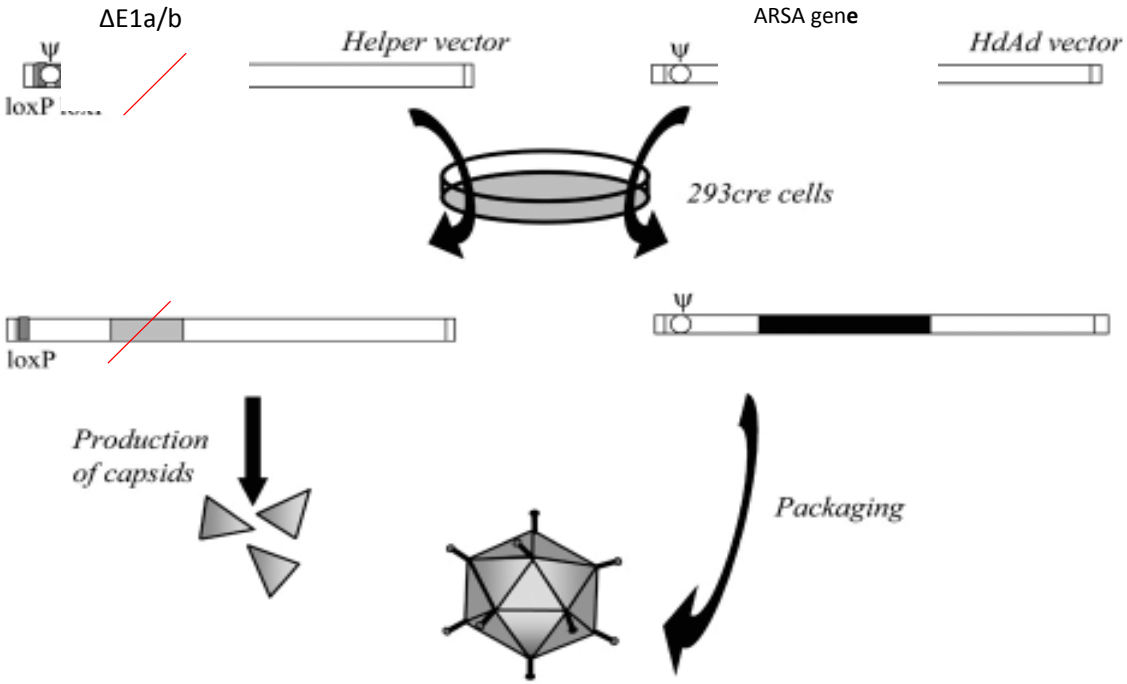


HDAd virus cassette



Helper vector cassette

ARSA: 5420 bp



THERAPEUTIC APPROACHES

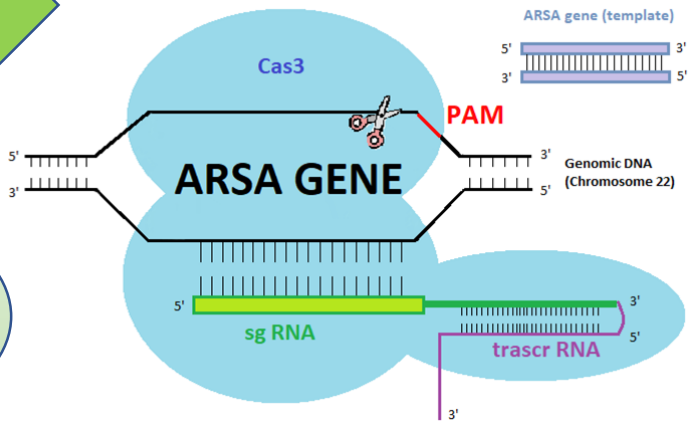
Bone Marrow and Umbilical Cord Blood Transplantation

***Ex-vivo* gene therapy: Bone Marrow Transplantation**

Enzyme Replacement Therapy (ERT)

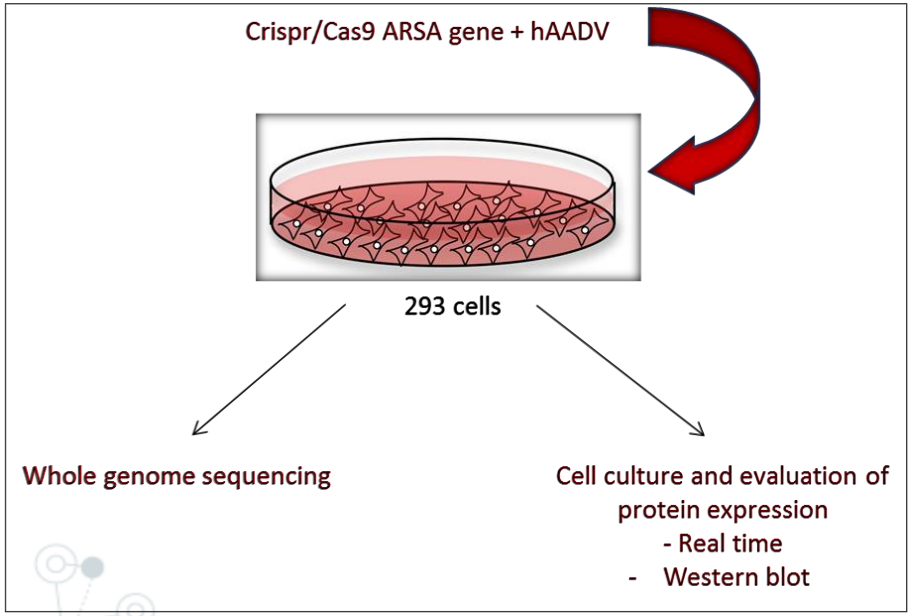
Adeno-associated Virus-mediated CNS gene therapy

Replacement of ARSA gene via CRISPR/Cas9 in bone marrow and ombelical cord cells

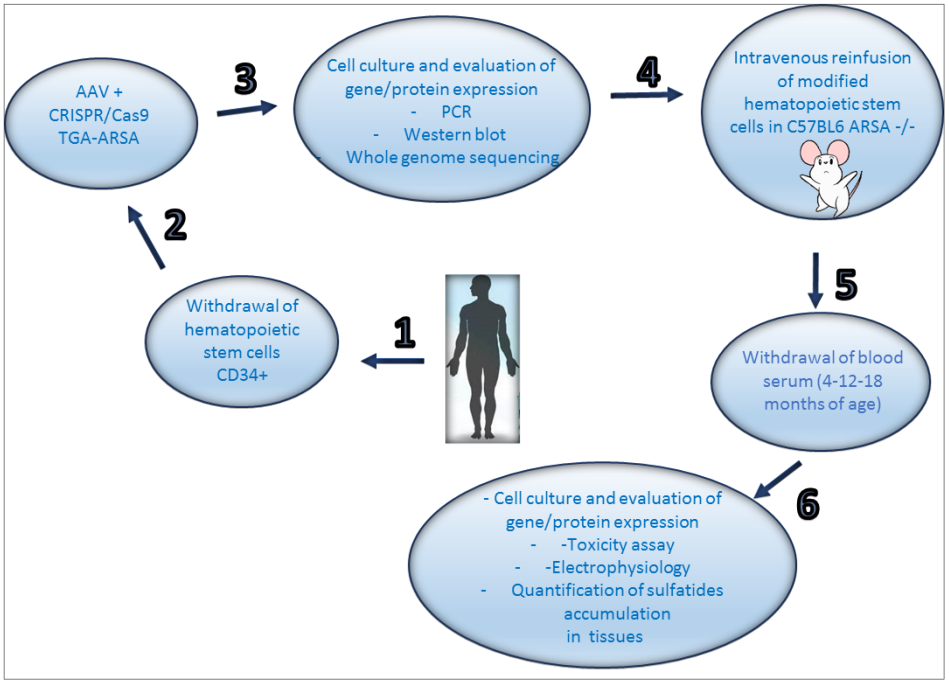


EXPERIMENTAL PLAN:

in vitro

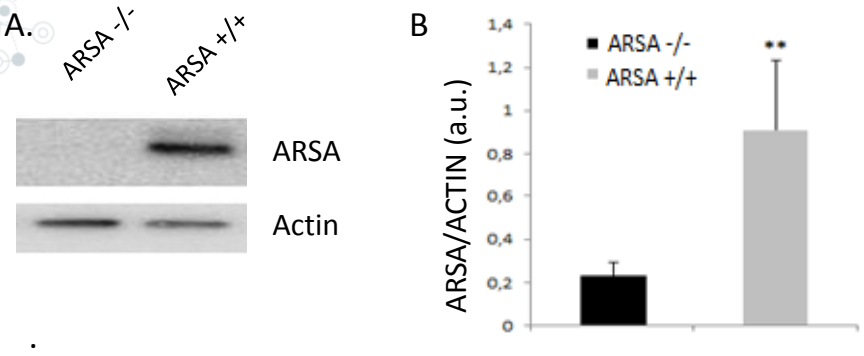


ex vivo/in vivo



EXPECTED RESULTS

in vitro experiment - 293T



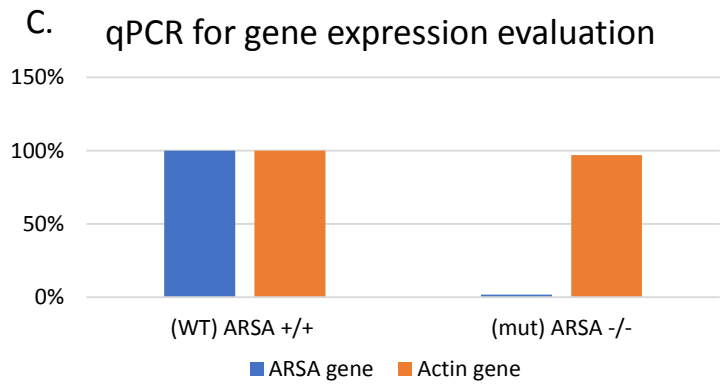
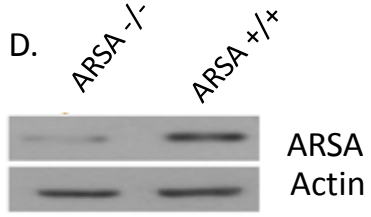


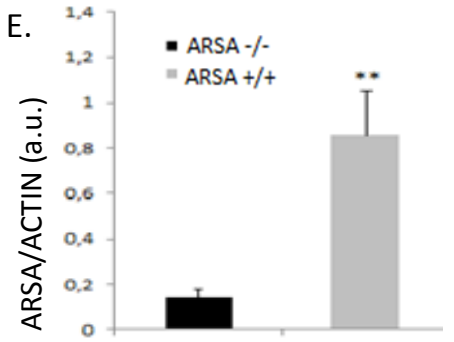
Fig. A) Western Blot Analysis of ARSA protein in lysates from 293T cells to test the overexpression of ARSA protein via CRISPR/Cas3 ARSA transgene activation;

Fig. B) Densitometry analysis of Wester blots, performed in four independent experiments via T-Student Test **p<0,01 (n=4) shows an increase in ARSA protein's levels;

Fig. C) qPCR for gene expression evaluation;

ex vivo experiment - hHSC





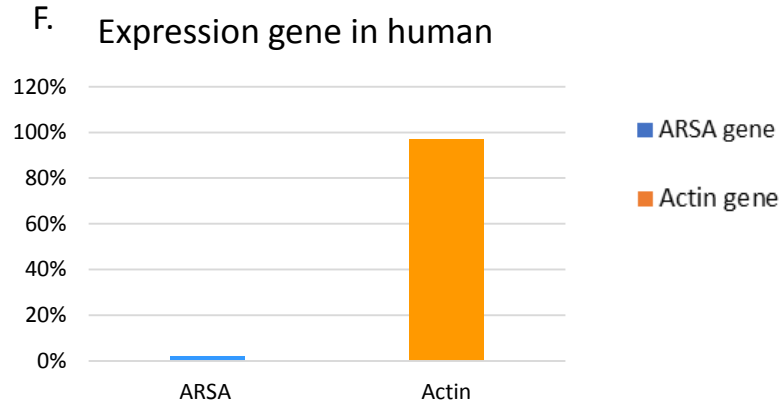


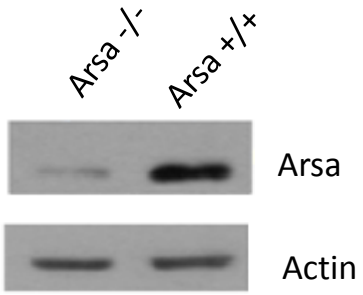
Fig. D) Western Blot Analysis of ARSA protein in lysates from bone marrow's human HSC, both in pathological and CRISPR/Cas 3 ARSA gene treated conditions;

Fig. E) Densitometry analysis of Wester blots, performed in five independent experiments via T-Student Test **p<0,01 (n=5) shows an increase in ARSA protein's levels;

Fig. F) qPCR for gene expression evaluation;

EXPECTED RESULTS
C57BL6 ARSA -/- mice experiments, *in vivo* (1)

A.



B.

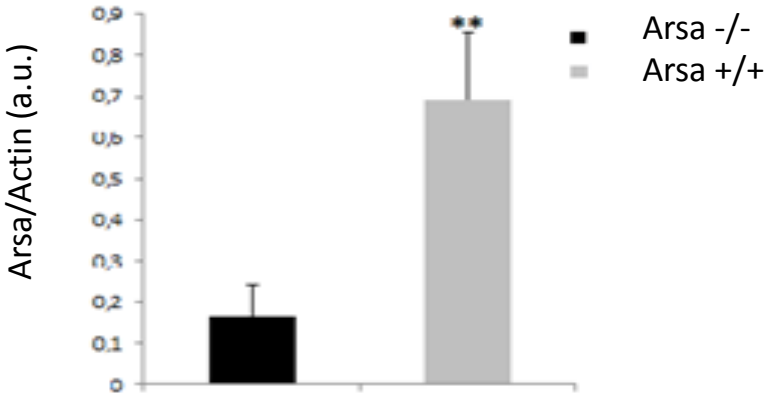


Fig. A) Western Blot Analysis of ARSA protein in lysates from ARSA-knockout mice and CRISPR/Cas3 ARSA gene treated mice;

Fig. B) Densitometry analysis of Wester blots, performed in three independent experiments via T-Student Test **p<0,01 (n=3) shows an increase in ARSA protein's levels.

C.

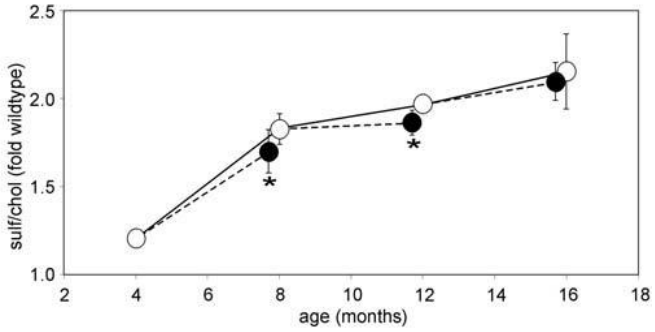


Fig. C) *(Closed circles: rhASA-treated MLD mice; Open circles: mock-treated* MLD mice)*

ERT had not the potential to reduce brain storage at any age, but merely to counteract the continuous accumulation of sulfatide during the treatment period.

* Another treatment option

EXPECTED RESULTS
C57BL6 ARSA -/- mice experiments, *in vivo* (2)

1.

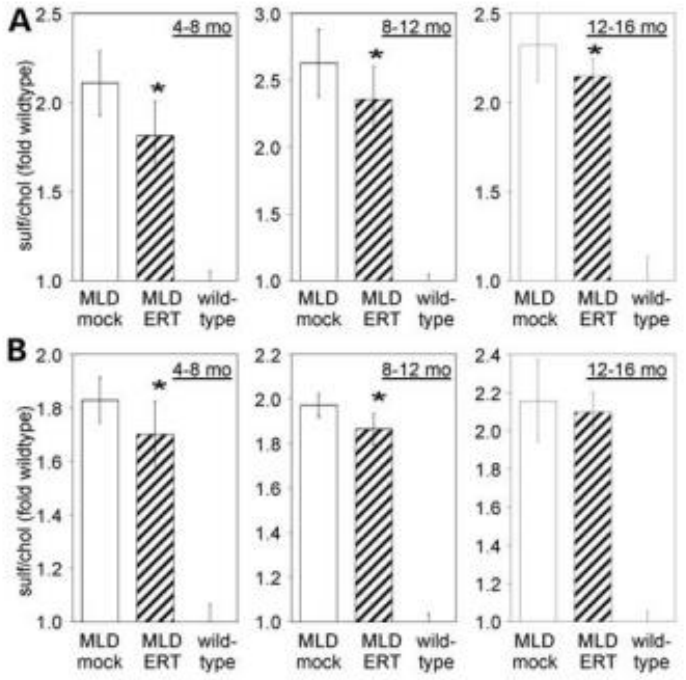


Fig.1) Toxicity assay: Sulfatide storage and sulfatide clearance. Sulfatide levels in (A) the kidney and (B) brain of mock-treated MLD mice (open bars), rhARSA-treated MLD mice and wild-type controls were determined by TLC, normalized to cholesterol levels and expressed as multiples of the mean wild-type levels.

2.

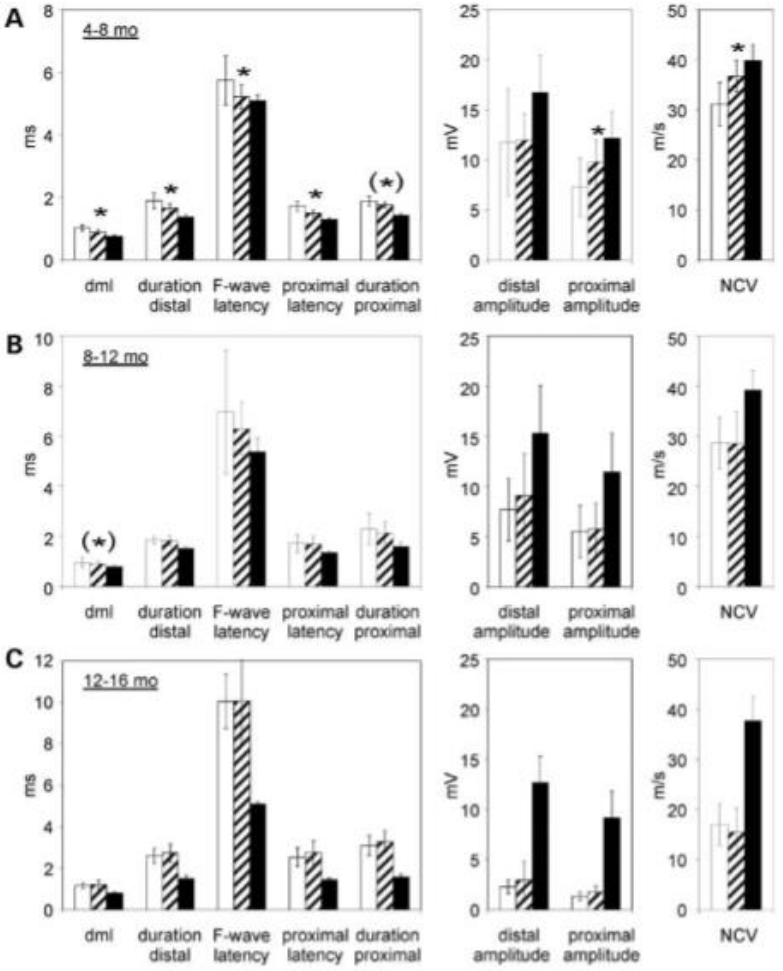
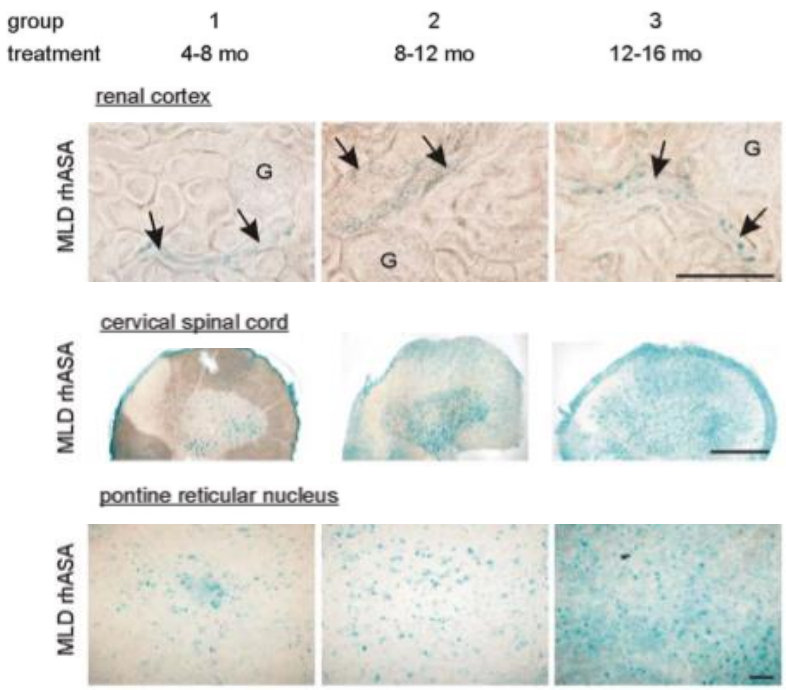


Fig. 2) Neurography of the sciatic nerve.CMAPs were elicited by proximal or distal stimulation of the sciatic nerve.The indicated electrophysiological parameters were compared between mock-treated MLD mice (open bars), rhASA-treated MLD mice (scattered bars) and wild-type controls (closed bars). Differences between mock-treated MLD mice and wild-type controls were significantly different for all 24 comparisons shown in the figure (P , 0.05, not indicated)

EXPECTED RESULTS
C57BL6 ARSA -/- mice experiments, *in vivo* (3)

1.



(G, glomerulus. Arrows in the lower panel point to TALs of the loop of Henle which are devoid of storage in rhASA-treated MLD mice).* Reduced sulfatide accumulation – gradual loss of Alcian blue marker.

ERT (Enzyme Replacement Treatment) = not result in a histologically detectable decline in storage material in the brain and spinal cord of
MLD mice at any age.
However, a decline in storage levels by 15.4% in group 1 (P ¼ 0.023) and by 11.0% in group 2 (P ¼ 0.005). In group 3, no statistically significant decline in sulfatide storage was detectable. *(data not shown)*

* TALs = Thick ascending limbal

Fig.1) Histology of sulfatide storage*(by incubation of tissue sections with Alcian blue)*

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PITFALLS AND SOLUTIONS

- Off target possibility?

  Presence of CNV evaluation

- Blood - brain barrier

  evaluation of other vectors
  (e.g. Lentiviral vectors or stem cells as vector for delivering?

- No stable vector integration for AAV

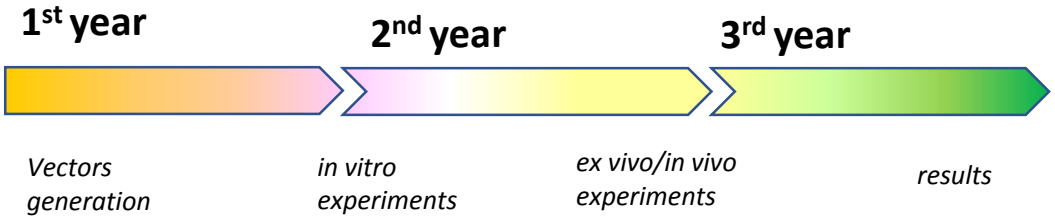
  Use of lentivirus could be better, but random integration possibility

- ARSA enzyme dosage could be introduce in prenatal diagnosis.

TIMELINE, MATERIALS AND COST OF THE PROJECT

	quantity	single cost	VAT tax (22%)	total amount
AAV6-CMV-Null Titer: 1x1013 GC/ml - *Vector Biolabs*	1	495,00 €	108,90 €	€ 604
AAV Transduction Kit – 50 reactions - *Antibodies-online.com*	1	907,00 €	199,54 €	€ 1.107
pCRIS-PITChv2-FBL plasmid - *Addgene*	3	65,00 €	14,30 €	€ 238
mice (WT) - *Charles River Laboratories, Inc.*	6	20,00 €	4,40 €	€ 146
B6N.129P2(CBA)-Arsatm1Gie/J - *The Jackson Laboratory*	12	130,00 €	28,60 €	€ 1.903
Stabulation cost (each mouse) - University facility	1000	1,50 €	0,33 €	€ 1.830
293T cell line human - *Sigma Aldrich*	4	299,50 €	65,89 €	€ 1.462
DMEM (10 x 500 ml) - *Gibco ThermoFisher Scientific*	10	229,00 €	50,38 €	€ 2.794
FBS (1 x 500 ml) - *Gibco ThermoFischer Scientific*	6	538,00 €	118,36 €	€ 3.938
L-Glutamine (200 mM) - *Gibco ThermoFisher Scientific*	6	24,53 €	5,40 €	€ 180
Penicillin-Streptomycin (10,000 U/mL) 100ml - *Gibco ThermoFisher Scientific*	6	35,43 €	7,79 €	€ 259
Western blot kit (ONE-HOUR Western™ Standard Kits 5 assays) - *Genscript*	10	66,00 €	14,52 €	€ 805
Western blot antibodies ARSA/ASA and Actin - *abcam*	5	658,00 €	144,76 €	€ 4.014
QuantiFast Multiplex RT-PCR Kit (80) - *Qiagen*	4	213,00 €	46,86 €	€ 1.039
AccuTaq™ LA DNA Polymerase High fidelity Taq enzyme - *Sigma-Aldrich*	7	226,00 €	49,72 €	€ 1.930
Flask, Falcon, Eppendorf, Sterile Pipette, Filter Tips (different amounts)	-	2.500,00 €	550,00 €	€ 3.050
NGS reaction average cost per sample - *Illumina seq*	1000	30,00 €	6,60 €	€ 36.600
Molecular biology basic/general reagents and homemade solutions	-	1.800,00 €	396,00 €	€ 2.196
Molecular biology laboratory instruments and furniture	-	8.000,00 €	-	€ 8.000
Extra, Packaging and shipping costs (average overall amount)	-	-	-	2.000,00 €

Time of the project: **36 months**



Total cost per year **€ 74.095**

(without the salary cost of researchers)