Shotgun Proteomics Reveals New Therapeutical Targets in Nephropathic Cystinosis

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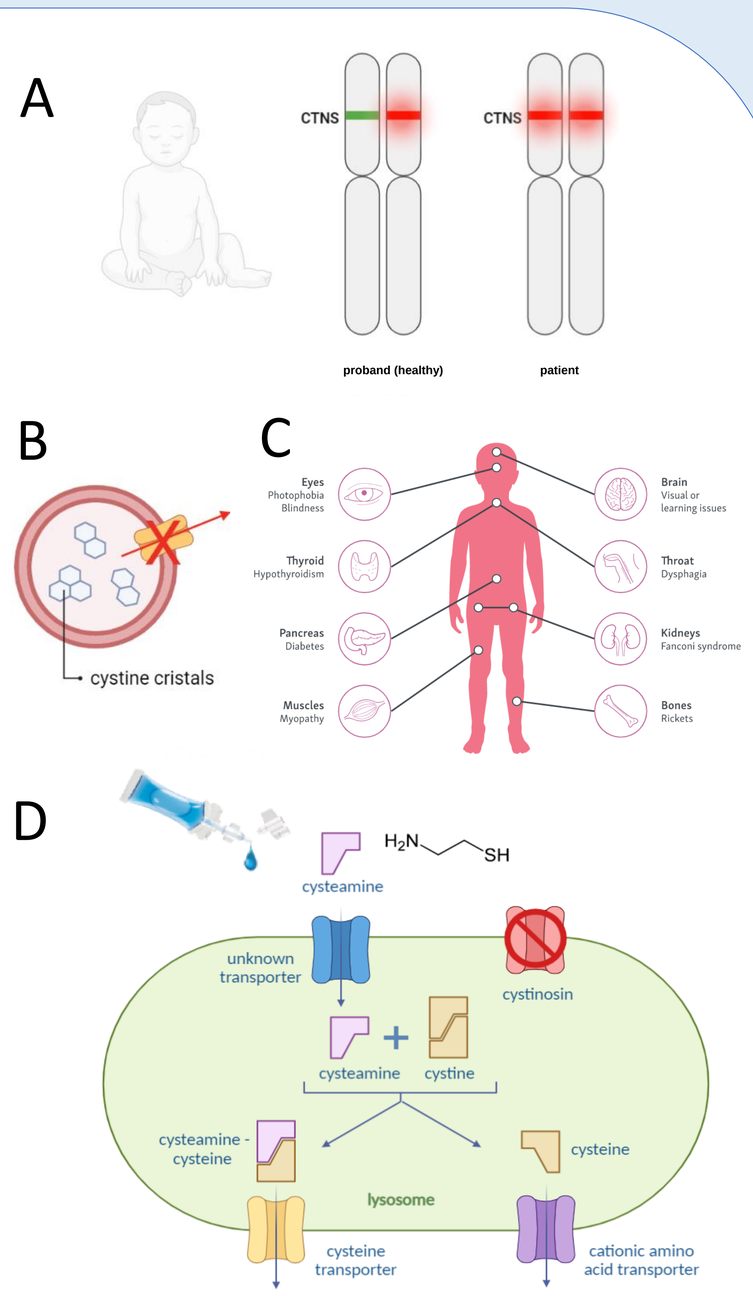
INTRODUCTION: What is nephropathic cystinosis?

Nephropathic cystinosis is a rare autosomal recessive metabolic disease due to mutations in the *CTNS* gen codifying for cystinosin (1), a lysosomal symporter **(A).** It is characterized by the accumulation of cystine crystals in lysosomes **(B)** causing, among other symptoms, end-stage renal damage and blindness in pedriatic patients **(C).** Currently, there is no cure, and the only palliative treatment is cysteamine (CTA), a highly reactive aminothiol that depletes cystine from lysosomes thanks to the formation of the cysteamine-cysteine complex (2), which uses the lysine amino acid transporter to facilitate lysosomal expulsion **(D).** However, CTA presents several limitations including the lack of cure for the disease, a wide plethora of adverse effects, and the complexity of the indicated treatment for life (3).

Recent findings indicate that the intra-lysosomal accumulation of cystine alters key processes such as phagocytosis, redox balance, and autophagy, causing a molecular imbalance that, to date, has not been characterized in detail (4,5).



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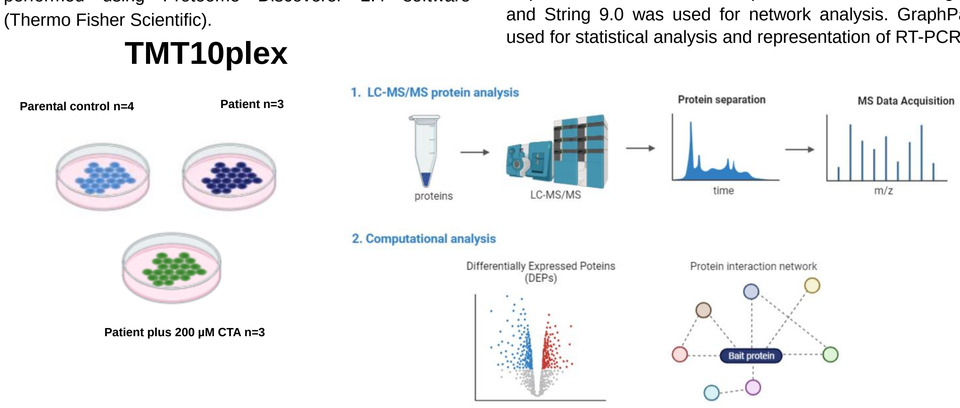
METHODS: How can we know more about nephropathic cystinosis at the cellular level.

We have performed a Data-Dependent Acquisition quantitative proteomic study (TMT10plex) including skin fibroblasts from patients with nephropathic cystinosis (n=3) and parental controls healthy (parents) (n=4) to obtain the proteomic signature of the patients versus the parental controls as well as the protein modulation by the treatment with cysteamine (48h at 200 µM). Orthogonal validation was done by Real-time-PCR. Proteomic data was acquired in a Orbitrap-Elite platform and Xcalibur 3.1 software (Thermo Fisher Scientific) was used for data acquisition, raw file generation, inspection of the chromatography profile and confirmation of the labelling of the peptides. After that, protein identification and quantification were performed using Proteome Discoverer 2.4 software (Thermo Fisher Scientific).

Peak lists were generated with a precursor signal-to-noise ratio of 1.5, and default settings were used to search the latest Human UniProtKB/SwissProt selease with the Sequest algorithm. The enzyme specificity was set to trypsin, and one missed cleavage was tolerated.

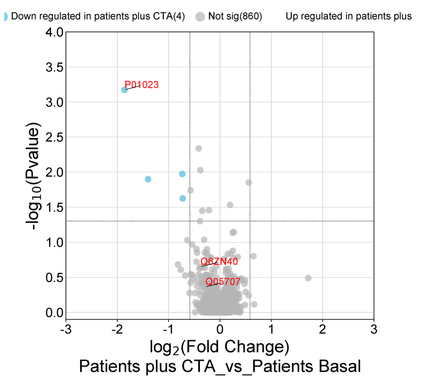
TMT-labeling and carbamidomethylation of cysteines were set as fixed modifications, whereas oxidation of methionines and N-terminal acetylation were set as variable modifications. The precursor ion mass tolerance was set to 7 ppm, and the product ion mass tolerance was set to 0.06 Da. A decoy database search was performed to determine the peptide false discovery rate (FDR) with the Target Decoy PSM (peptide-spectrum matches) Validator Module. Quantification was performed using a Quantification Module, and normalization was performed against the total peptide amount. A 1% peptide FDR threshold was applied.

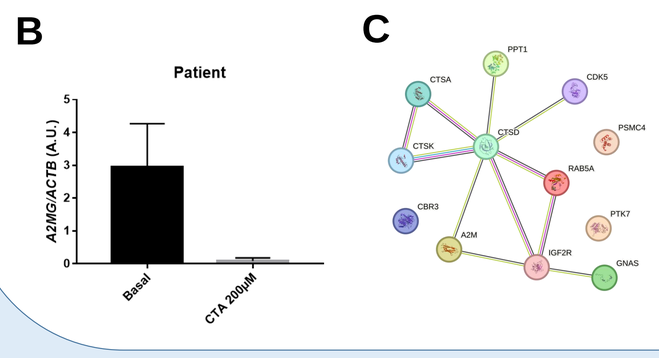
Representation of modulated proteins was done using SRPlot and String 9.0 was used for network analysis. GraphPad was used for statistical analysis and representation of RT-PCR data.

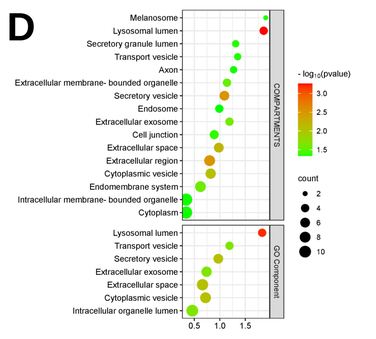


RESULTS: Proteomic signature of CTA-treated cystinotic cells.

In order to study the effect of CTA we also compared cells from patients in basal condition *vs.* cells of patients treated for 48h with 200µM CTA (mimicking the plasma concentrations in treated patients) We observed that the upregulation of A2M is reversed **(A, B)** in patients when cells are incubated with cysteamine. Other proteins modulated by cysteamine **(C)** stand out that are implicated in renal pathology and lysosomal maturation **(D)**.

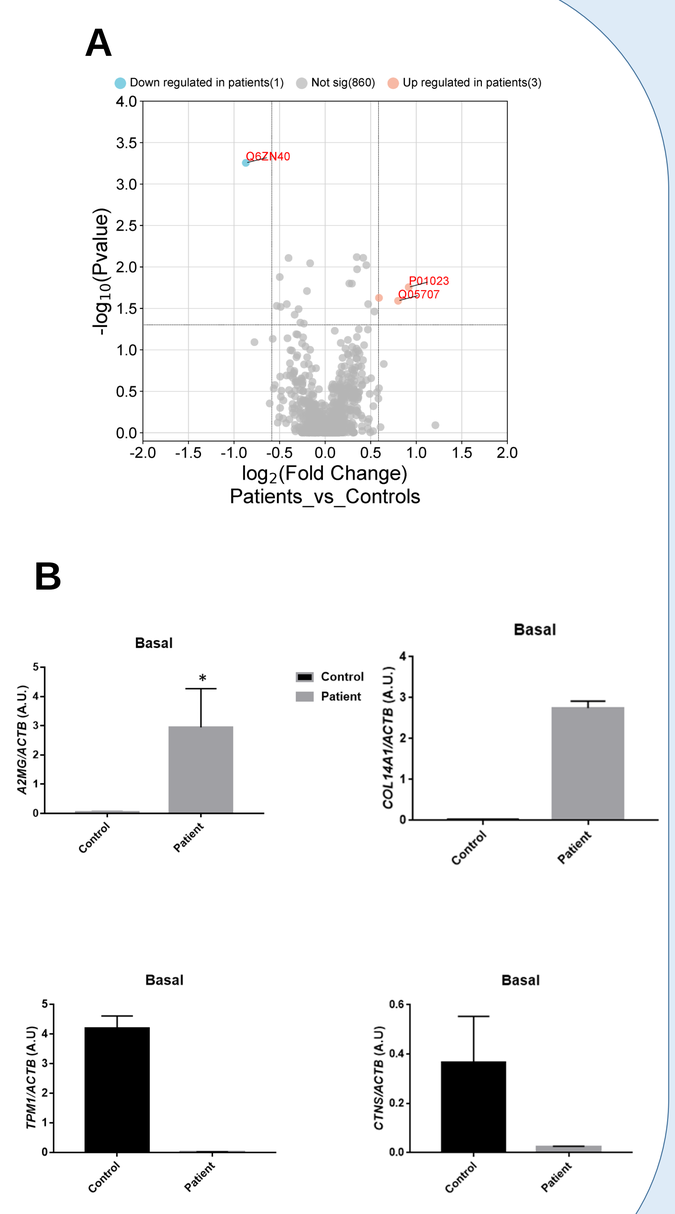






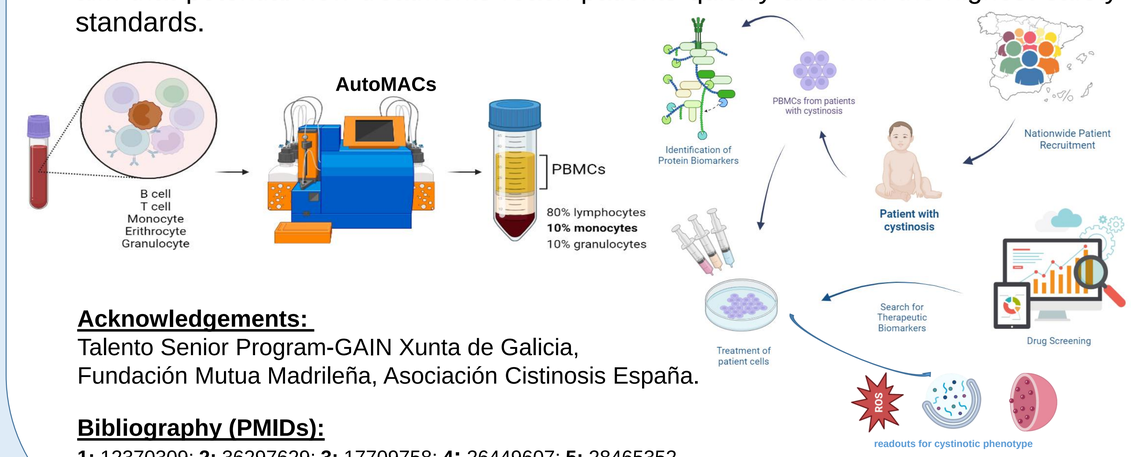
RESULTS; Proteomic signature of cystinosis patients *versus* healthy parental controls.

We observed that a group of proteins appears significantly ($p \leq 0.05$) altered in patients compared to their healthy parental controls **(A)**. The most upregulated protein in patients is alpha-2-macroglobulin (**A2M**, P01023; Fold change 1.89), a protein that has recently been proposed as biomarker of nephrotic syndrome. A2M is a protease inhibitor protein that plays an important role in processes such as regulation of endocytosis, immune cell proliferation, and monocyte-mediated inflammation (6). Macrophage binding of receptor-recognized forms of A2M (a2M*) significantly increases cAMP, CREB, and activated CREB (7). An increase in A2M has been related, for example, to higher levels of collagen since A2M inhibits the proteases that degrade it (8). Interestingly, in our proteomic study we have detected Collagen Type XIV Alpha 1 Chain (Q05707; Fold change= 1.74) also significantly increased in patients. On the other hand, Tropomyosin-1 (Q6ZN40; Fold change: 0.54) is the most down-regulated protein in patients The proteomic data have been corroborated by Real-Time PCR as well as the *CTNS* deficiency in patients **(B)**.



Conclusions and work in progress.

Our results suggest an important role of endocytosis, immune cell proliferation, and monocyte-mediated inflammation in nephropathic cystinosis. *Stop Cystinosis* is a private-funded project with the aim of using SWATH-MS technology to identify altered molecular pathways in the disease. As a cellular model, we are isolating peripheral blood mononuclear cells (PBMCs) from patients with cystinosis and healthy parents as isogenic controls. Based on this information we will identify new potential therapeutic targets. The PBMCs will again be used for high-throughput screening with selected batteries of small molecules with known pharmacological activity and their effect on the cystinotic phenotype of the cells will be studied, paying special attention to the therapeutic repositioning of already marketed drugs, with the aim that potential new treatments reach patients quickly and with the highest safety standards.



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Bibliography (PMIDs):

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