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CRF6 – Grant No. CRFF-2018-008A : "Evaluation of Ctns$^{-/-}$ mice protection by oral supplementation with basic amino-acids : focus on kidneys with extension to another colony" Fourth and last semestrial DDUV report *–March 1, 2020 to August 31, 2020*

This fourth and last grant-period has been seriously impacted by Covid-19. Despite the difficulties, we could pursue two objectives related to the evaluation of diet supplementation by natural, inexpensive dibasic L-amino-acids (dAAs) as potential flexible adjuvant therapy in nephropathic cystinosis : (i) evaluation of long-term benefits and potential liver toxicity of *primary* dAA treatment in cystinotic mice (with UCSD); (ii) submission and approval of amendment of ongoing contract on *secondary* dAA treatment in cystinotic mice (started after 6 mo; with OPBG, Roma), to allow parallel study in cystinotic rats (also with OPBG).

Previous data.

As our main task for this grant, we pursued our collaboration with Drs R. Mak and W. Cheung by the comparison between WT and *Ctns*$^{-/-}$ female mice at 9-months, each in three experimental groups :control, L-lysine- or L-arginine-treated (dAAs). This *"preventive" (primary), long-term study* (2-to-9 mo) aimed to address, with adequate statistical power of large numbers, the potential of dbAAs to prevent cystine accumumation and protect cystinotic kidney structure and function. At the last CRF meeting, Dr Mak reported that, in their colony treated with food from Research Diet Inc, 5x-dAAs apparently fail to offer *long-term* protection against nephropathic cystinosis, as compared to cystinotic mice receiving control diet where 5x-"L-lysine" (see below) or 5x-L-arginine are substituted by isonitrogenous glycine (state-of-the-art control, because of no known competition for endocytosis in short-term rat studies, but same nitrogen supplementation). However, questions have been raised on the validity of this control because of (i) unexpected death of a significant fraction of *Ctns*$^{-/-}$ mice at UCSD receiving "control" diet; and (ii) favorable comparison in *short-term* studies at DDUV and OPBG of dAA-treatments *vs control diet without glycine supplemention* revealed significant protection of dAA-treated cystinotic mice. Moreover, (iii) scrutinizing the diets provided by the reputable Research Diet Inc, we realized that instead of incoroporating plain L-lysine as requested, diet was supplemented by L-lysine-HCl, in other words caused an acid stress to cystinotic mice that could potentially compound the interpretation of lysine effects *per se*. For these three reasons, we are happy that a new study is running with OPBG, with control diet without glycine supplementation (as would control patients feed) and strict use of plain5x- L-lysine (without HCl); to also be compared with 5x-D-lysine and 5x-D-arginine.

New data.

As promised, we performed carefull immunofluorescence studies in *kidneys from 4 mice of the three Ctns*$^{-/-}$ *mice experimental groups at UCSD*, sacrificed at 9 months, selected to span the min-max range of swan-neck frequency (see Fig 1A, adapted from third interim report). In keeping with the conclusions of Drs Mak and Cheung studies and our previous morphometry of swanneck lesions, we could not find obvious difference in expression level and subcellular positioning of megalin and NaPi-IIa, as representative markers of endocytic receptors and solute transporters (Figs 1 & 2). We thus decided to stop further analysis of these two markers. However, we noted that the size of lysosomes appeared distinctly smaller in the 2 of the 4 kidneys from 5x-arginine-fed mice, which also displayed fewer swan-necks. We will not miss to further test this encouraging trend in the ongoing dAA mice study in collaboration with OPBG.

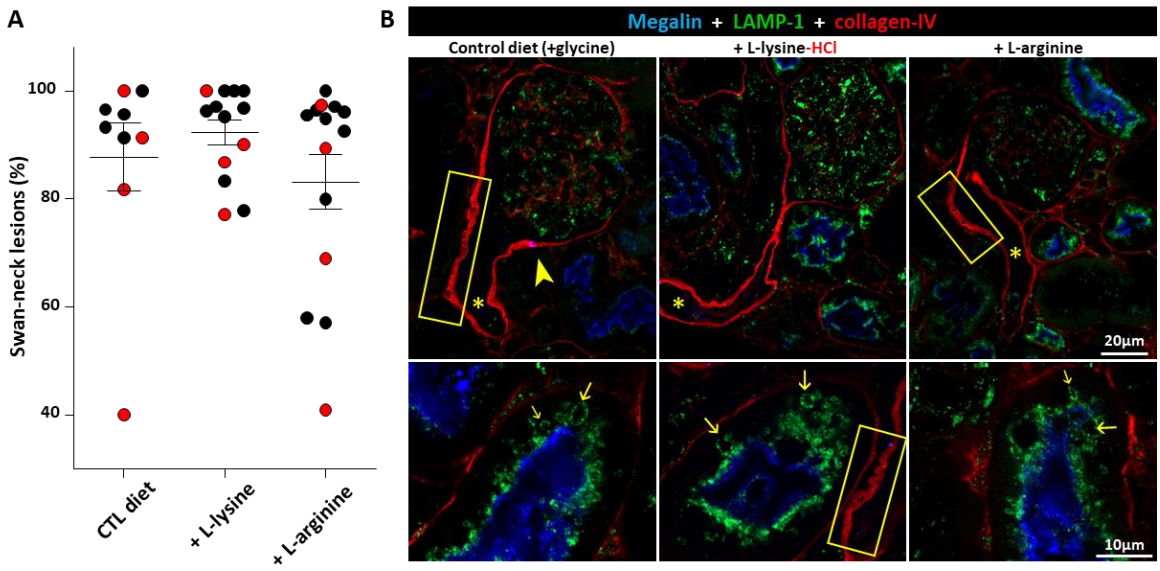

Figure 1 : A. Identification of four kidneys per group selected to span the min-max range of swan-necks, with suggestive relevance for lysosomal changes under L-arginine diet (see main text). It is hoped that development of a method to quantify longitudinal extension of proximal tubule atrophy (as a continuous variable) beyond the glomerulo-tubular junction (as all-or-none variable) in ongoing new study will allow to stretch min-max range in all experimental groups. **B. Triple confocal immunofluorescence for megalin (endocytic receptor, cyan); LAMP-1 (lysosomes, green) and type IV-collagen (basement membrane, red) in *Ctns*$^{-/-}$ kidneys from UCSD colony at 9 months of age after long-term primary « control diet » (isonitrogenous glycine), « L-lysine » (5x-L-lysine-HCl) or 5x-L-arginine.** Upper panels are centered on glomerulo-tubular junctions with typical swan-neck lesions characterized by atrophy of proximal tubular-cells (asterisks, « empty lumen ») and sharp transition (large arrowhead) from Bowman's capsule where basement membrane is of normal thickness, to tubular basement membrane of much increased thickness and irregularities including duplication and discontinuities (yellow boxes). Lower panels are centered on more distal sections of the proximal tubules, to appreciate normal expression and apical positioning of megalin, abundance of lysosomes, some of which moderately enlarged (small arrows) or greatly enlarged and/or distorted lysosomes as we and other previously described. As shown by these representative images, there was no detectable qualitative difference between the three experimental groups of this study for megalin, but much fewer large lysosomes were definitely seen in the two arginine-treated kidneys which had the lowest proportion of swan-necks. Of note, in each group, atrophy extension seemed lesser than in our previous study (Janssens et al, JASN, 2018).

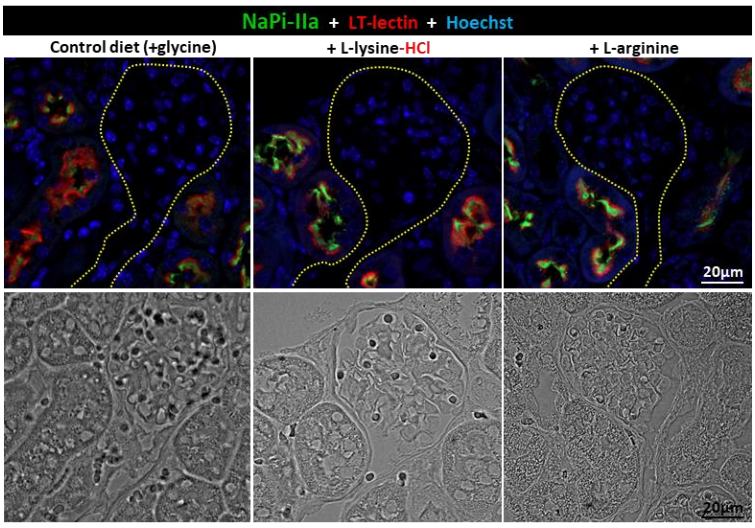

Figure 2 : Triple confocal (immuno) fluorescence for NaPi-IIa (solute transporter, green); *Lotus tetragonolobus* lectin (LT-lectin, apical membrane, red) and Hoechst (nuclei, cyan) in *Ctns*$^{-/-}$ kidneys from UCSD colony at 9 months of age after long-term primary « control diet » (isonitrogenous glycine), « L-lysine » (5x-L-lysine-HCl) or 5x-L-arginine. Upper panels are fluorescence images. In swan-neck profiles, delineated by yellow dotted lines, notice full disappearance of NaPi-IIa and LT-lectin labelling, two molecular hallmarks of PTC atrophy. Outside swan-neck lesions, NaPi-IIa and LT-lectin signals are well-preserved. Lower panels are phase-contrast images of the same fields. Notice the contrast between altered (left panel) or empty structures (central and right panel) at swan-necks, contrasting with preserved (sub)cellular structures of glomeruli and more distal proximal tubules. As shown by these representative images, there was no detectable qualitative difference between the three experimental groups of this study.

Our UCSD colleagues had mentioned unusual liver findings ("they referred to these as cysts"). Of note, these were limted to WT mice or "control" diet. As also promised, we thus explored liver toxicity by two

approaches. First, we measured *liver cystine levels* in the three experimental groups among *Ctns*$^{-/-}$ and WT mice. As shown by Fig 3, there was no difference in the low levels of the three WT groups, as expected. In contrast, we confirmed the predicted increase in liver cystine content of *Ctns*$^{-/-}$ under control diet, and no change under 5x-L-arginine; but noted a surprising three-fold increase under 5x-L-lysine-HCl.

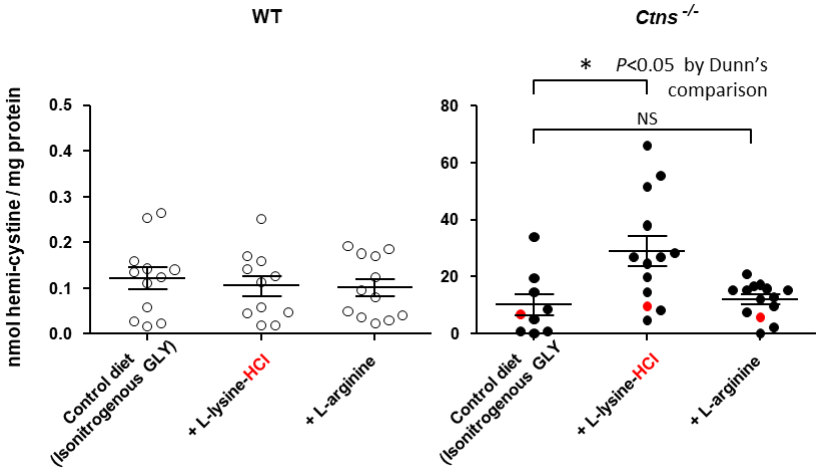

Figure 3 : Effect of diets on total liver cystine content at 9 months of age. At left, the very low levels of cystine in WT liver (around 0.1 ng hemi-cystine/mg tissue protein) are not affected by 5x-dAAs. At right, levels are increased by about 100-fold in *Ctns*$^{-/-}$ liver in the UCSD colony under « control diet » (about 10 ng hemi-cystine/mg tissue protein, yet values are six times lower than at the DDUV colony as reported by Janssens et al, JASN, 2018; whether this reflects incomplete protection during overseas shipment or further processing of exceedingly large samples cannot be assessed). Of note, levels are not detectably affected by chronic 5x-L-arginine oral supplementation (2-to-9 months). Thus, altough « control » diet with isonitrogenous supplementation may be questioned, L-arginine appears safe by this test. Whereas we currently assume that 5x-L-arginine oral supplementation could compete for LRP2-megalin at the apical domain (brush border) of kidney proximal tubular cells, this data would suggest that it does not detectably inhibit LRP1-scavenger receptor of Küpffer cells where cystine crystals are essentially concentrated in *Ctns*$^{-/-}$ liver. In surprising contrast, levels are further increased by about three-fold (about 29 ng hemi-cystine/mg tissue protein; *P*<0.05) upon long-term primary 5x-L-lysine-HCl supplementation (2-to-9 months). Whether (or to what extent) this unexpected increase of liver cystine accumulation is related to the unwanted concomitant acid load should be clarified by new study at OPBG with plain L-lysine. Red symbols identify representative mice used for Fig4.

Second, we performed immunoperoxidase staining for glutamine synthase in paraffine liver sections from all groups as well-known marker of metabolic stress related to the urea cycle, expected to be challenged under increased nitrogen supplementation, and of which arginine is a metabolic step ("ornithine-citrulline-arginine"). This data is illustrated by Fig 4. Of note, this test focuses on the centrilobular zone of liver lobules. Counterstaining the same material, allowed to survey overall liver histology. An expert liver pathologist, blind of exerimental group, noticed systematic anomalies in all WT livers; thus we disregarded this comparison. For *Ctns*$^{-/-}$ livers, data were generally scored as "normal" and we did not find systematic change in relation to the diet. Thus, despite higher cystine content, L-lysine-HCl did not induce detectable liver histopathological alteration.

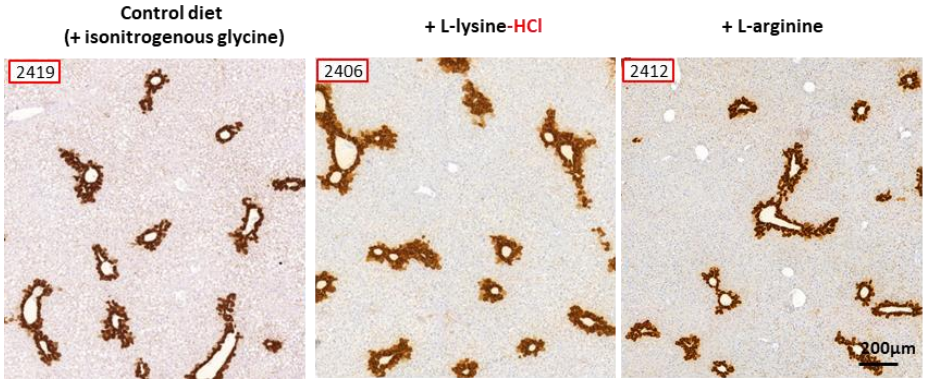

Figure 4 : Glutamine synthase as stress metabolic marker in *Ctns*$^{-/-}$ liver. Representative views of several paraffine liver sections after immunoperoxidase staining. Notice intense labeling limited to one or two hepatocyte layers surrounding centrilobular veins, as normally found. There was no consistent difference between the three experimental groups. Alterations in metabolic diseases include thickening (multilayering) of labeled zones.

In conclusion, this study of primary (early), long-term (2-to-9 months) treatment of cystinotic mice by 5x-dAAs showed overall good general tolerance but appeared disappointing as it did not evidence long-term kidney protection by dAAs, nor muscle strenthening. It however disclosed two major limitations : poor relevance of glycine supplementation for control; and unwanted acid load by the L-lysine-HCl diet. This unwanted acid stress could have contributed to the moderately impaired renal function in this group, as reported by Drs Mak and Wilson, or increased liver cystine. These two problems have been corrected for the ongoing study of secondary treatment. We also decided with Dr Rega to add measurements of liver cystine in this new study. As pointed out by Dr Mak at the last CRF meeting, L-arginine does not promote body building without exercice, of which encaged mice are deprived.

We also have hint of a possible correlation in the arginine-treated *Ctns*$^{-/-}$ kidneys between much fewer swannecks and much fewer enlarged/distorted lysosomes. This aspect will be pursued in the new study.