Development of a Functional Assay to Diagnose Creatine Transporter Deficiency using UPLC-MSMS system.

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Background

Creatine transporter deficiency (CTD) is an X-linked disorder caused by variants in the creatine transporter gene (SLC6A8) and characterized by intellectual disability, failure to thrive, speech delay, autistic-like behavior, and seizures. Affected patients have increased urine creatine/creatinine ratio and pathogenic variants in the *SLC6A8* gene. Genetic testing can miss variants outside the coding region of the gene or detect missense variants of uncertain significance (VUSs). In such cases, a functional assay can be used to confirm the diagnosis. We are developing a new assay to measure creatine transport in fibroblasts using a stable isotope rather than radioactive creatine.

Methods

LC-MSMS:

d3- and d5-Creatine were measured by UPLC-MS/MS (Waters ACQUITY UPLC I-Class System and Xevo TQ-XS Mass Spectrometer) after dry down of the intra-cellular extract and derivatization with butanolic HCl. Creatine was chromatographically resolved using a reverse phase column (Acquity UPLC BEH C18, 1.7µm, 2.1 x 100 mm, with a 0.2 µm in-line pre-column filter) and detected using tandem mass spectrometry.

Cell Culture and Creatine Transport :

Fibroblasts from patients and normal controls were cultured in DMEM supplemented with 15% fetal bovine serum and were seeded in 24-well plate and grown to confluence. On the day of the experiment, the cells were depleted of intracellular amino acids by incubation for 60 min in Earle's balanced salt solution containing 5.5mM D-glucose and supplemented with 0.5% bovine serum albumin. Cells were then incubated for the indicated time in the presence of d5-creatine (20 µM) at 37°C. The transport reaction was stopped by rapidly washing the cells 3 times with ice-cold 0.3 M Urea. Non-saturable creatine transport was measured in the presence of 2 mM creatine. Intracellular creatine was extracted with 0.3 ml of ice-cold ethanol followed by internal standard d3-creatine (400nM) addition. Values were normalized for the protein content of each well and intracellular water content and expressed as nmol/ml cell water/h. Saturable creatine transport was calculated by subtracting transport in the presence of excess (2mM) substrate from total transport.

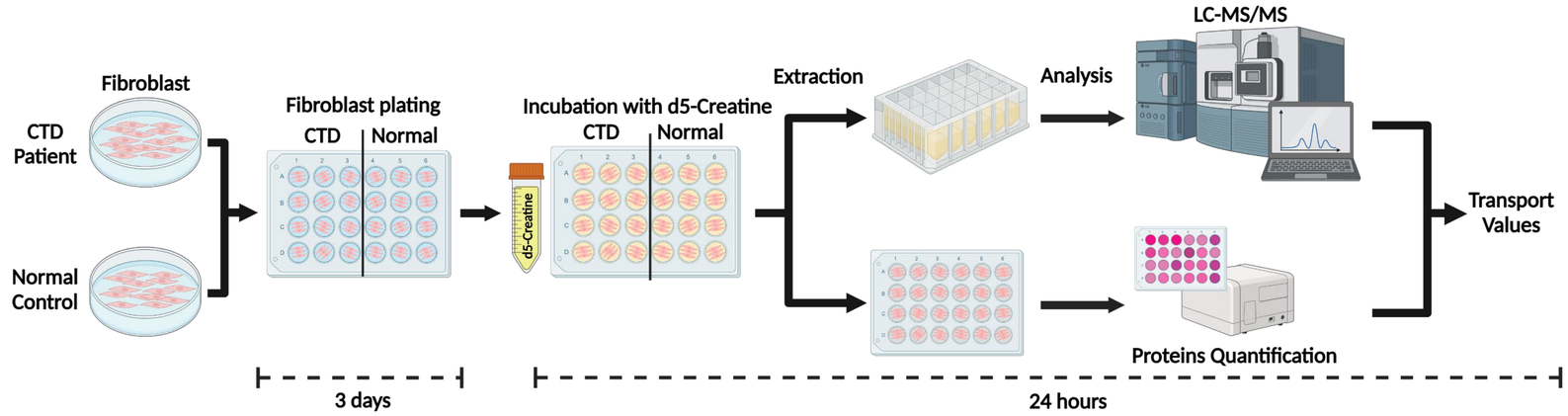

Figure 1. Workflow of Creatine Transport Functional Test

Results

- The molecular transitions selected for d3-creatine was 191 > 93, and for d5-creatine was 193 > 95 (Figure 2 A and 2B). The d3- and d5-creatine showed a retention time of 2.55 and 2.54 min respectively (Figure 2C and 2D).
- In normal cells, net creatine uptake (total uptake minus the non-saturable uptake measured in the presence of 2mM unlabeled creatine) was linear for up to 2 h and then declined slightly at the 4 h time point Figure 3B).
- The patient's fibroblasts demonstrated markedly impaired activity that was reduced to 1% and 1.5% of control (Figure 4A and 4B)

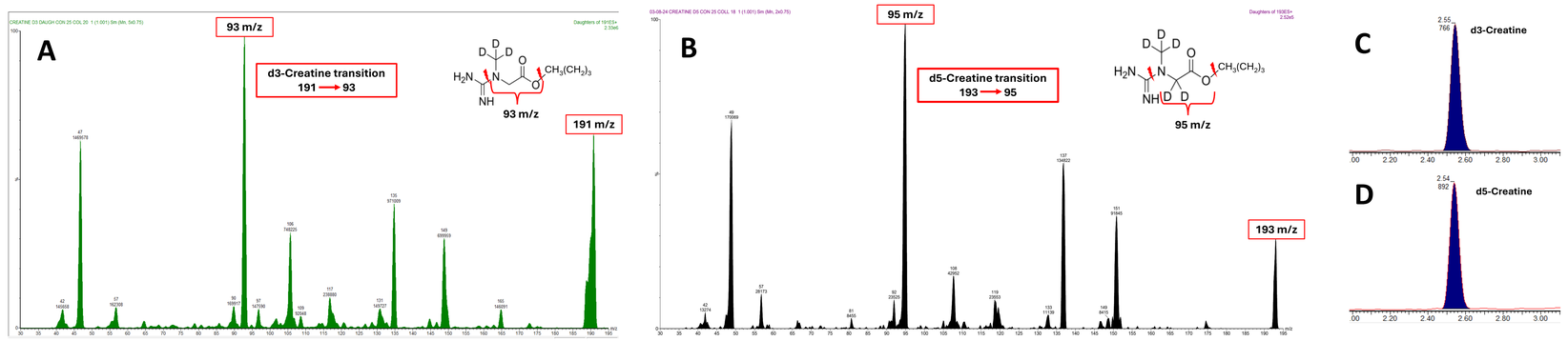

Figure 2. d3-creatine and d5-creatine spectrums (A, B) and chromatograms (C,D)

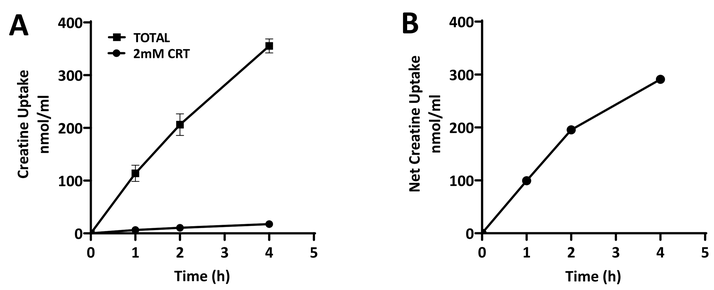

Figure 3. Time-course of creatine transport in human fibroblasts. Creatine (20 µM) uptake was measured for the indicated time in cells from normal control in the absence (TOTAL) or presence of excess (2 mM) creatine (A). Points are averages of triplicates ± SD. Net uptake was calculated by subtracting uptake in the presence of excess creatine from total uptake (B).

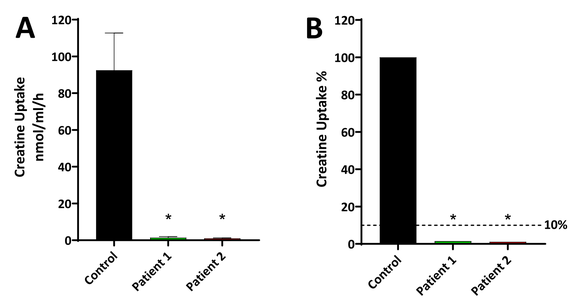

Figure 4. Creatine uptake in human fibroblasts. Creatine transport was measured for 2 h in cells from two CTD patients and five unrelated controls (A). Creatine uptake % was represented as percent of control (B). CTD diagnosis threshold was set at 10% of control. Points are averages of triplicates ± SD. *P<0.01 versus n=5 controls.

Conclusion

This is a reliable method for measuring creatine transport in fibroblasts. It allows for the confirmation of the diagnosis of CTD and possibly the identification of CTD patients with residual transport activity.

Acknowledgments

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