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ORIGINAL ARTICLE

Serum global metabolomics profiling reveals profound metabolic impairments in patients with MPS IIIA and MPS IIIB

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Abstract The monogenic defects in specific lysosomal enzymes in mucopolysaccharidosis (MPS) III lead to lysosomal storage of glycosaminoglycans and complex CNS and somatic pathology, for which the detailed mechanisms remain unclear. In this study, serum samples from patients with MPS IIIA (age 2-9 yr) and MPS IIIB (2-13 yr) and healthy controls (age 2-9 yr) were assayed by global metabolomics profiling of 658 metabolites using mass spectrometry. Significant alterations were detected in 423 metabolites in all MPS III patients, of which 366 (86.5%) decreased and 57 (13.5%) increased. Similar profiles were observed when analyzing data from MPS IIIA and MPS IIIB samples separately, with only limited age variations in 36 metabolites. The observed metabolic disturbances in MPS III patients involve virtually all major pathways of amino acid (101/150), peptide (17/21), carbohydrate (19/23), lipid (221/325), nucleotide (15/25), energy (8/9), vitamins and co-factors (8/21), and xenobiotics (34/84) metabolism. Notably, detected serum metabolite decreases involved all key amino acids, all major neurotransmitter pathways, and broad neuroprotective compounds. The elevated metabolites are predominantly lipid derivatives, and also include cysteine metabolites and a fibrinogen peptide fragment, consistent with the status of oxidative stress and inflammation in MPS III. This study demonstrates that the lysosomal glycosaminoglycans storage triggers profound metabolic disturbances in patients with MPS III disorders, leading to severe functional depression of virtually all metabolic pathways, which emerge early during the disease progression. Serum global metabolomics profiling may provide an important and minimally invasive tool for better understanding the disease mechanisms and identification of potential biomarkers for MPS III.

Keywords Lysosomal storage diseases · MPS iii · Global metabolomics profiling · Mass spectrometry · Metabolic impairments

Introduction

Mucopolysaccharidosis (MPS) III is group of 4 (A-D) autosomal recessive disorders, each caused by mutations in a gene that encodes a specific lysosomal enzyme that is essential in the stepwise degradation of heparan sulfate (HS) glycosaminoglycans (GAGs) (Neufeld and Muenzer 2001). Each enzyme deficiency leads to the accumulation of HS-GAGs within the lysosomes. The disease causing alleles of each MPS III are highly polymorphic, with broad variations in mutations identified (Yogalingam and Hopwood 2001). The lysosomal storage of HS-GAGs leads to similar clinical manifestations across all 4 types of MPS III, with predominantly severe progressive neurological disorders. Somatic manifestations of MPS III do occur in all patients, but are mild, relative to other forms of MPS. Infants appear normal at birth, but develop profound neurological manifestations at the age of 2–4 years, including developmental delay, reduced cognitive capacity, hyperactivity, and ultimately death (Yogalingam and Hopwood 2001).

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The primary pathology of MPS III has been characterized as the accumulation of HS-GAGs in lysosomes in cells of virtually all tissues/organs, especially in the central nervous system (CNS) involving both neuronal and non-neuronal cells (Bhaumik et al. 1999; Li et al. 1999; Neufeld and Muenzer 2001). In addition, our recent studies demonstrate widespread profound neuropathology in the peripheral nervous system (PNS) (Fu et al. 2012), indicating that neuropathological manifestation affects the entire nervous system. While the detailed mechanisms of pathology, especially neuropathology of MPS III, are not yet well understood, numerous studies have reported cascades of complex secondary pathological events in the CNS, including broad metabolic impairments (Bhaumik et al. 1999; McGlynn et al. 2004; Woloszynek et al. 2007; McCarty et al. 2011; Wilkinson et al. 2012; Fu et al. 2017), neuroinflammation (Tamagawa et al. 1985; Li et al. 2002; Ohmi et al. 2003; Villani et al. 2007; DiRosario et al. 2009; Wilkinson et al. 2012; Duncan et al. 2015; Martins et al. 2015), oxidative stress (Villani et al. 2007; Villani et al. 2009; Martins et al. 2015), autophagy (Ryazantsev et al. 2007; Settembre et al. 2008), and neurodegeneration (Tamagawa et al. 1985; Kurihara et al. 1996; Ginsberg et al. 1999; Li et al. 2002; Hamano et al. 2008; DiRosario et al. 2009; Ohmi et al. 2009; Ohmi et al. 2011; Winder-Rhodes et al. 2012; Naughton et al. 2013; Martins et al. 2015).

No definite treatment is currently available for MPS III, and therapies have been limited to palliative treatment. Significant therapeutic advancements have been made for treating lysosomal storage diseases (LSDs), such as hematopoietic stem cell transplantation (HSCT), recombinant enzyme replacement (ERT) and gene therapy (Valayannopoulos and Wijburg 2011). MPS III (A-D) disorders are not amenable to currently approved systemic ERT or HSCT, which have shown somatic benefits, since the blood-brain-barrier (BBB) precludes effective CNS access to either recombinant enzyme or enzyme produced by transplanted hematopoietic stem cells. Alternative intrathecal ERT clinical trials are ongoing, targeting the CNS disorders in patients with MPS I, II and IIIA, and require indefinite repetitive administration. Gene therapy has been considered an ideal approach for treating LSDs because of the potential for long-term endogenous production of recombinant enzymes without the need to treat every cell. Numerous virus-mediated gene therapy studies, mostly designed to restore the missing enzyme activity, have shown varying degrees of correction of lysosomal storage in vitro and in vivo in LSD animal models, using various viral vectors (Sands and Haskins 2008; Byrne et al. 2012). Recombinant adeno-associated viral (rAAV) has been a favored vector for gene delivery because it is non-pathogenic, with demonstrated long-term expression in the CNS and periphery (High and Aubourg 2011; Byrne et al. 2012). The recent finding of trans-BBB neurotropism in AAV9 offers an effective solution for CNS gene therapy, showing great potential for the treatment of LSDs and other neurological diseases (Duque et al. 2009; Foust et al. 2009; Marmiroli et al. 2009; Foust et al. 2010; Fu et al. 2011). A single systemic delivery of rAAV9 vectors can lead to global CNS and widespread somatic restoration of enzyme activity, correction of lysosomal storage pathology, and functional neurological benefits in mice with MPS IIIB or IIIA (Fu et al. 2011; Ruzo et al. 2012; Fu et al. 2016). These studies have led to FDA approval for Phase 1/2 gene therapy clinical trials in patient with MPS IIIA (NCT02716246, ongoing) and MPS IIIB (to be initiated). Autologous hematopoietic stem cell gene therapy approaches have also been shown to be beneficial for the treatment of MPS III in mouse models (Langford-Smith et al. 2012; Sergijenko et al. 2013).

As therapeutic development advances and more therapies for MPS become available, the lack of accessible biomarkers will be a critical challenge for therapeutic assessment. Urinary GAGs and specific lysosomal enzymes have been the only biomarkers for MPS, although recent studies identified serum heparin cofactor II-thrombin (HCII-T) as a biomarker for MPS I, II and III (Randall et al. 2008), dipeptidyl peptidase IV (DPP-IV) for MPS I, II, III, IVA and VI (Beesley et al. 2009). More recently, disease-specific non-reducing end carbohydrates have demonstrated their potential as biomarkers for MPS (Lawrence et al. 2012). However, there are currently no specific biomarkers for MPS III corresponding to disease severity or therapeutic responsiveness. Our recent study of serum global metabolomics profiling showed profound impairments involving broad metabolic pathways in MPS IIIB mice (Fu et al. 2017). Importantly, these metabolomics abnormalities responded well to the previously demonstrated effective gene therapy approach by a single systemic rAAV9-hNAGLU delivery (Fu et al. 2011; Naughton et al. 2013), suggesting the biomarker potential of serum metabolomics profiles for MPS IIIB (Fu et al. 2017). Given that the MPS III mouse model resembles the human diseases in virtually all aspects, and the close similarity of MPS disorders, we hypothesized that the profound abnormalities of serum metabolomics profiles would also occur in humans with MPS IIIB, as well as other MPS disorders. Assessing the biomarker potential of metabolomics profiles becomes more significant as ERT and gene therapies for MPS IIIA and MPS IIIB are being tested in clinical trials. In this study, serum global metabolomic profiling was performed using mass spectrometry (MS) to identify metabolic impairments in patients with MPS IIIA and MPS IIIB.

Materials and methods

Serum samples and study subjects

Serum samples were obtained from patients with MPS IIIA and MPS IIIB enrolled in a natural history study at Nationwide Children's Hospital (NCH), which was approved by NCH

Institutional Review Board (IRB).(Truxal et al. 2016) Controls serum samples were obtained from age matched healthy individuals via BioServe (Beltsville, Maryland USA).

Serum sample process for metabolomics analyses

Serum samples were assayed for metabolites at Metabolon Inc. (Durham, NC). All serum samples were stored at −80 °C prior to analyses. At the time of the analyses, serum samples were processed to extract metabolites using the automated MicroLab STAR® system (Hamilton Company) and Metabolon's standard methods via a methanol extraction, allowing maximum recovery of small molecules. Each resulting serum sample extract was divided into five equal fractions: two for analysis by two separate reverse phase (RP) ultrahigh performance liquid chromatography-tandem mass spectroscope (UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for metabolomics analysis.

Global metabolomic profiling

Non-targeted global metabolomics profiling (Metabolon, Inc.) of human serum extract was performed using RP/UPLC-MS/MS methods with positive ion mode ESI, RP/UPLC-MS/MS with negative ion mode ESI, and HILIC/UPLC-MS/MS with negative ion mode ESI, as previously described (Sekula et al. 2016), resulting in the identification and quantification of 658 compounds of known structural identity. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slighted between methods but covered 70–1000 m/z. The information output from the raw data files was automatically extracted and archived by Metabolon Laboratory Information Management System (LIMS). Metabolites were identified by comparison to metabolomics library entries of purified standards. Data are presented as fold of change in 1) MPS III patients vs. healthy controls, as well as 2) MPS IIIA patients vs. healthy controls, 3) MPS IIIB patients vs. healthy controls, or 4) MPS IIIA at older age (A3 + A4) vs. young age (A1 + A2): >1.0 = increase, <1.0 = decrease.

Statistical analysis

Statistical analyses of log-transformed metabolomics data were performed using Welch's two-sample *t*-test, two-way ANOVA and the False Discovery Rate (FDR). Significance of *t*-test and ANOVA analyses was defined as $p \leq 0.05$ and approaching significance as $0.05 < p < 0.10$). Significance of FDR analysis was also defined as $q \leq 0.05$.

Results

We performed global metabolomics profiling for 658 metabolites to analyze serum samples from 25 patients with MPS III, including MPS IIIA ($n = 15$, age 2.1–9.2 years) and MPS IIIB ($n = 10$, age 2.3–13.7 years). Serum samples from healthy individuals ($n = 23$, age 2–9 years) were used as controls. Within each group, samples were further divided into 3–4 subgroups based on subject ages. Table 1 shows the demographic distribution of the study subjects.

Mass spectrometry reveals decisive MPS III serum metabolomic profiles

Serum global metabolomics profiling by mass spectrometry was performed to assess metabolic abnormalities in patients with MPS IIIA or MPS IIIB. Principal component analysis (PCA) (Fig. 1) and heat map analyses (Supplementary Table S1) of the 658 metabolites showed clear separation of serum metabolomics profiles in MPS III patients from those of matched healthy individuals. Further, Random Forest classification analysis showed a predictive accuracy of 100%, comparing structurally named serum metabolites in MPS III patients and healthy controls (Supplementary Fig. S1). This clear division that was also observed in MPS III mice (Fu et al. 2017), indicating that the detected metabolomic abnormalities are highly likely be MPS III-associated, and that global metabolomic profiling has potential for the identification of pathological metabolomic signatures of MPS III disease in humans.

Serum global metabolomics profiling revealed profound metabolic impairments in patients with MPS III disorders

Using MS, we detected significant differences ($p \leq 0.05$ and $q \leq 0.05$) in 423 metabolites (64%) in MPS III patients, when comparing data from all MPS III patients ($n = 25$) to that of healthy controls ($n = 23$) (Table 2, Supplementary Table S1). The majority of the significantly altered serum metabolites in MPS III patients were reduced compared to healthy controls, with 366 (86.5%) decreased and 57 (13.5%) increased

(Table 2, Supplementary Table S1). Importantly, the detected metabolomic abnormalities in MPS III patients were associated with broad (virtually all) metabolic pathways, involving the metabolism of amino acids (101/150), carbohydrates (19/23), lipids (221/325), peptides (17/21), nucleotides (15/25), energy (8/9), cofactors/Vitamins and (8/21) and xenobiotics (34/84) (Table 2, Supplementary Table S1). Furthermore, group analyses using two-way ANOVA further confirmed the observed serum metabolomics alterations in MPS III patients (Supplementary Table S1). Notably, our data showed similar serum metabolomics profiles between MPS IIIA and MPS IIIB patients (Table 2, Supplementary Table 1, Figs. 2, 3, 4 and 5). Further, when comparing profiles from 2 to 5 year-old (A1 + A2, n = 10) with 6–9 year-old (A3 + A4, n = 5) MPS IIIA patients, significant differences were detected in only 39 of these metabolites ($p \leq 0.05$), and the estimated rate of false discovery among these 39 altered metabolites was calculated to be 80% ($q \leq 0.08$) (Supplementary Table S1). These data indicate that the lysosomal HS-GAG storage triggers profound metabolic suppression in MPS III patients, which emerges early, before 2 years of age. The following data are based on all MPS III subjects combined, given the similarity of serum metabolomics profiles of MPS IIIA and MPS IIIB patients at different ages (Table 2, Supplementary Table S1).

Table 1 Demographic distribution of study subjects

Subjects		Age (yr)	Gender
MPS III (n = 25)		**6.4 ± 3.1****	
MPS IIIA (n = 15)		5.0 ± 1.9**	M/F = 9/6
A1	1	2.1	F
	2	2.6	M
	3	2.8	M
	4	3.4	F
A2	1	4.2	F
	2	4.2	M
	3	4.7	M
	4	4.7	F
	5	5.0	F
	6	5.2	F
A3	1	5.8	M
	2	6.0	M
	3	6.4	M
A4		8.0	M
		9.2	M
MPS IIIB (n = 10)		8.6 ± 3.0**	M/F = 8/2
B1	1	2.3	F
B3	1	5.8	M
	2	6.5	F
	3	7.8	M
B4	1	9.3	M
	2	9.3	M
	3	9.6	M
	4	13.7	M
	5	11.2	M
	6	11.0	M
Controls (n = 23)*		**5.5 ± 2.5****	**M/F = 11/12**
C1 (n = 6)		2–3 (2.6 ± 0.4)	M/F = 3/3
C2 (n = 6)		4–5 (4.5 ± 0.6)	M/F = 3/3
C3 (n = 6)		6–7 (6.8 ± 0.4)	M/F = 3/3
C4 (n = 5)		8–9 (8.5 ± 0.5)	M/F = 2/3

*Non-MPS individuals with no major medical concerns

**Age (mean ± SD)

Notably, our combined data showed reductions in numerous serum amino acid metabolites in MPS III patients, including 8 essential (8/9), 4 conditionally essential (4/6), and 5 non-essential (5/5) amino acids (Fig. 2, Supplementary Table S1), indicating broad functional defects ranging from absorption, to biosynthesis of amino acids. There were also significant disturbances in branched-chain amino acid pathways (Supplementary Fig. S2). Importantly, we also detected significant reductions in serum amino acids and derivatives that can function as key neurotransmitters or components critical in neurotransmitter metabolism (Fig. 3a, c). Of these, dopamine 4-sulfate became undetectable (down 6.7-fold) and dopamine 3-O-sulfate decreased 5.9-fold (Fig. 3a), suggesting severe impairment of the dopamine pathway. Significant decreases were also observed in multiple serum metabolites that are considered to be critical for neurological functions, such as N-acetylaspartate (NAA), N-acetylalanine, 3-methoxytyrosine, and taurine (Fig. 3b, Supplementary Table S1).

Among the significantly reduced serum carbohydrate metabolites in MPS III patients, there were a majority of the known essential components of protein and lipid glycosylation

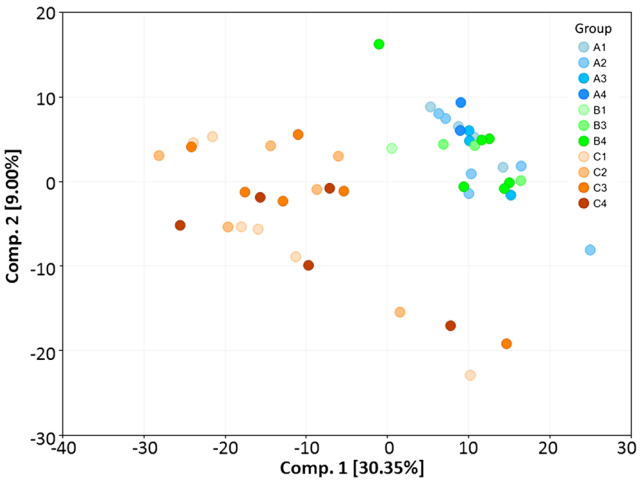


Fig. 1 Metabolomics impairments in patients with MPS III. Serum samples from MPS III patients and healthy control subjects were analyzed by global metabolomics profiling using mass spectrometry. Data were analyzed by principal component analyses. **A1–4:** MPS IIIA (n = 15, age 2.1–9.2 yr); **B1–4;** MPS IIIB (N = 10, age 2.3–13.7 yr); **C1–4:** Healthy controls (n = 23, age 2-9 yr)

Table 2 Broad metabolic impairments in MPS III patients

Pathways	No. metabolites assayed	No. metabolites changed ($P \leq 0.05$)*								
		All MPS III ($n = 25$)[#]			MPS IIIA ($n = 15$)			MPS IIIB ($n = 10$)		
		Total	Decrease	Increase	Total	Decrease	Increase	Total	Decrease	Increase
Amino acids	150	101	93	8	97	88	9	85	78	7
Carbohydrates	23	19	18	1	20	19	1	16	15	1
Lipids	325	221	181	40	207	173	34	178	144	34
Peptides	21	17	14	3	17	14	3	16	14	2
Nucleotides	25	15	14	1	13	11	2	15	14	1
Energy	9	8	8	0	8	8	0	9	9	0
Cofactors/Vitamins	21	8	6	2	5	3	2	8	6	2
Xenobiotics	84	34	32	2	30	30	0	28	28	0
Total	**658**	**423**	**366**	**57**	**397**	**346**	**51**	**355**	**308**	**47**

Serum samples from patients with MPS IIIA ($n = 15$) and MPS IIIB ($n = 10$) were assayed for 658 metabolites, using mass spectrometry.

* Changes in MPS III patients vs. healthy controls ($n = 23$).

[#] All MPS III (MPS IIIA + MPS IIIB)

pathways, such as fructose, erythronate, glutamate, mannitol/sorbitol, *N*-acetylglucosamine/*N*-acetylgalactosamine, and *N*-acetylneuraminate (Fig. 4**,** Table 2, Supplementary Table S1). One exception is serum mannose, which was significantly increased in MPS III patients (Fig. 4a, Supplementary Table S1). In addition, we also observed significant reductions in metabolites that are critical in glycolysis (pyruvate and associated) and TCA cycle (8/8) in serum from MPS III patients (Fig. 5, Supplementary Table S1), suggesting suppression in energy metabolism.

Significant abnormalities in lipid metabolites (221/325) were also detected, involving virtually all lipid classes measured, including fatty acids, lipids, sterols, steroid, and others, of which the majority (181) were reduced (Table 2, Supplementary Table S1). Notably, serum metabolites that were decreased the greatest in MPS III patients were 3 fatty acid amides (100%), of which, palmitic amide and linoleamide (18:2n6) each decreased 11-fold, while oleamide became undetectable (Supplementary Fig. S3, Supplementary Table S1). The majority of elevated serum metabolites (40/57) in MPS III subjects were lipids and derivatives, including long-chain fatty acids, polyunsaturated fatty acids, monoacylglycerols, and steroids. The broadest serum metabolite elevations were observed in the monoacylglycerol group, in which nearly all (15/16) measured compounds were increased in MPS III patients (Table 2, Supplementary Table S1), suggesting elevated lipase activity.

Other notable findings include significant elevation of serum cysteine metabolites in MPS III patients, including cystine, cysteine s-sulfate and oxidized cysteine-glycine (Fig. 6a, c, Supplementary Table S1), suggesting an oxidative stress status. We also detected a significant increase in fibrinopeptide A, fragment of the fibrinogen α-chain (Fig. 6b, Supplementary Table S1).

Discussion

This study demonstrates that lysosomal accumulation of HS-GAGs triggers profound metabolic impairments in patients with MPS IIIA and MPS IIIB, and serum global metabolomics profiling may provide an effective tool for better understanding the disease mechanisms and identification of potential biomarkers for disease status.

The primary pathology of MPS III is lysosomal accumulation of HS-GAGs in cells of virtually all tissues and organs, especially in the CNS, involving both neuronal and non-neuronal cells. While the detailed mechanisms of pathology of MPS III are not yet well understood, numerous studies have reported cascades of complex secondary pathological changes in MPS III, including metabolic impairments, involving GM2 and GM3 gangliosides, oxidative stress and altered energy balance (Bhaumik et al. 1999; Li et al. 1999; McGlynn et al. 2004; Villani et al. 2007; Woloszynek et al. 2007; Villani et al. 2009). Recently, using serum global metabolomics profiling, we detected severe progressive metabolic abnormalities in a mouse model of MPS IIIB, which are much more profound than previously described, and involve virtually all metabolic pathways, most of which are depressed (Fu et al. 2017). In the current study, we revealed profoundly impaired serum metabolomic profiles in patients with MPS III, which are similar to that observed in MPS IIIB mice (Supplementary Table S2) (Fu et al. 2017), supporting our hypothesis that serum global metabolomics profiles are highly

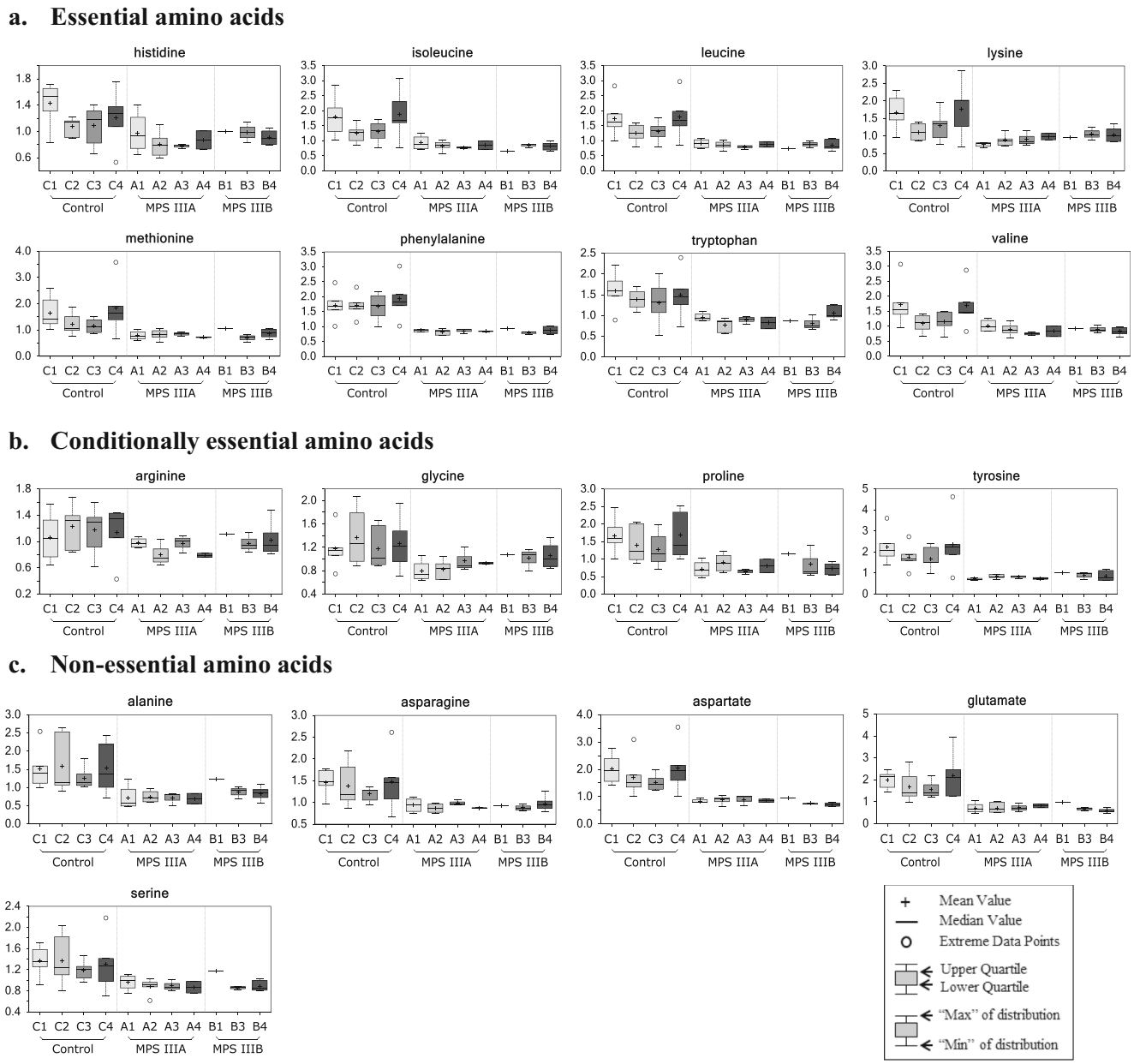
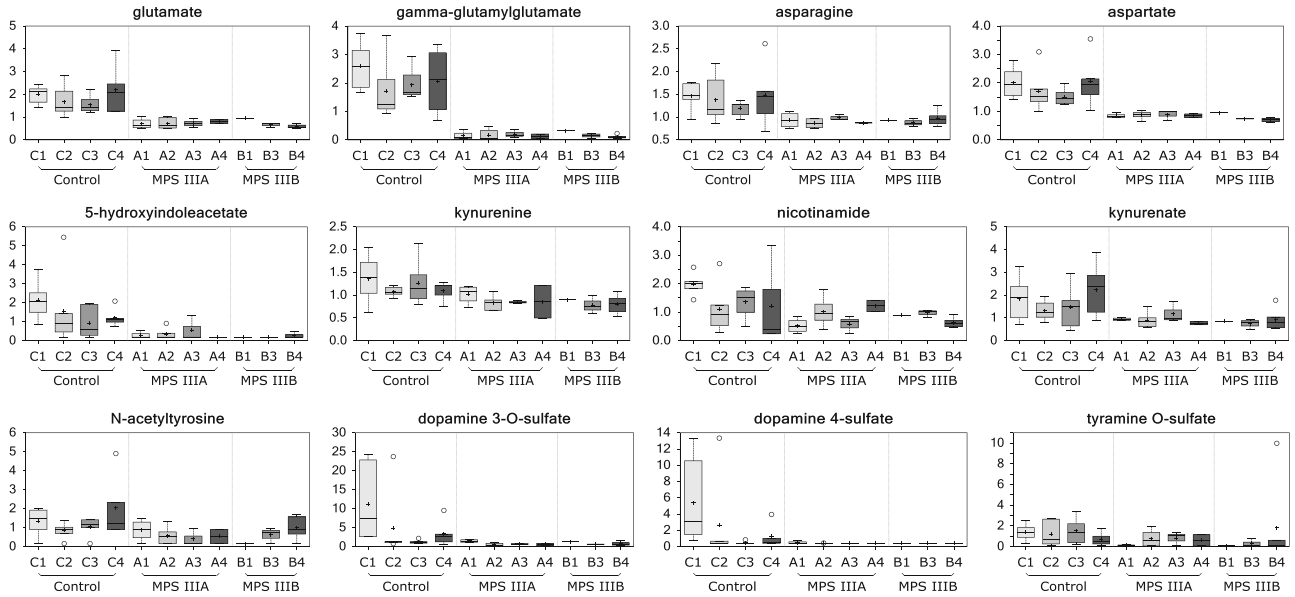
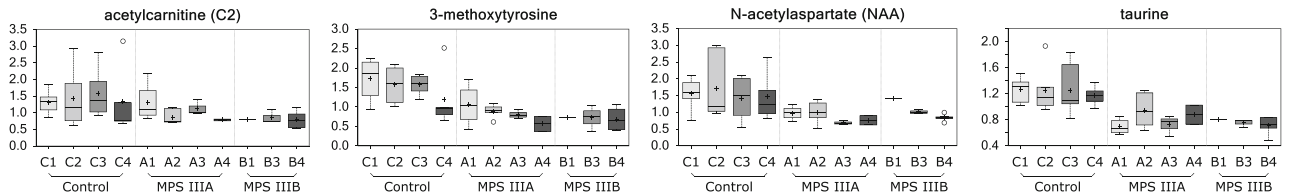


Fig. 2 Impairments in metabolism of essential, conditionally essential and non-essential amino acids in patients with MPS III. Serum samples from MPS III patients and healthy control subjects were analyzed by global metabolomics profiling using mass spectrometry. Data are presented as Scaled Intensity (**y-axis**) using box and whiskers plots, based on $p \leq 0.05$ MPS III vs. controls. **A1–4:** MPS IIIA ($n = 15$, age 2.1–9.2 yr); **B1–4;** MPS IIIB ($N = 10$, age 2.3–13.7 yr); **C1–4:** Healthy controls ($n = 23$, age 2-9 yr)

translatable between mouse models and humans, given the resemblance of MPS III mouse models to the human diseases (Bhaumik et al. 1999; Li et al. 1999; Neufeld and Muenzer 2001). Interestingly, the serum metabolomics profiles are largely similar between MPS IIIA and MPS IIIB (Supplementary Table S2), supporting the idea that the severe metabolic impairments result from lysosomal HS-GAG storage, rather than the specific gene defects. Further, our data indicate that the observed metabolic disturbance in MPS III patients begins before 2 years of age, since no meaningful age differences were detected.

As was observed in MPS IIIB mice, the detected metabolomic abnormalities in MPS III patients involve virtually all metabolic pathways of amino acids, peptides, lipids, carbohydrates, energy, nucleotides, cofactors/vitamins, and xenobiotics. Notably, similar to what were observed in MPS IIB mice(Supplementary Table S2) (Fu et al. 2017), the significantly altered serum metabolites in MPS III patients were predominantly reduced (86.5%), in agreement with the observation that lysosomal accumulation of HS-GAGs trigger severe impairments in widespread biochemical and molecular pathways (Li et al. 1999; McGlynn et al. 2004; Villani et al. 2007; Villani





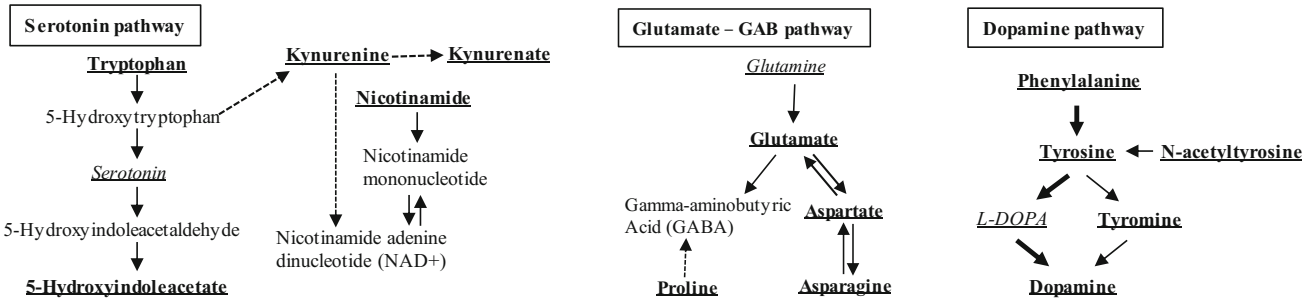


Fig. 3 Decrease in neurotransmitters and neuro-function associated metabolites in patients with MPS III. Serum samples from MPS III patients and healthy control subjects were analyzed by global metabolomics profiling using mass spectrometry. **a.** metabolomic comparison of neurotransmitters/derivatives; **b.** metabolomic comparison of neurofunction-associated metabolites; data presented as Scaled Intensity (**y-axis**) using box and whiskers plots, based on $p \leq 0.05$ MPS III vs. controls. **A1–4:** MPS IIIA (n = 15, age 2.1–9.2 yr); **B1–4;** MPS IIIB (N = 10, age 2.3–13.7 yr); **C1–4:** healthy controls (n = 23, age 2-9 yr). **c.** neurotransmitter pathways; metabolites; **bold and underlined metabolites:** $p \leq 0.05$ MPS III vs. controls; **underlined italic metabolites:** changes not significant $p > 0.05$; **Others:** not assayed

et al. 2009; Naughton et al. 2013; Duncan et al. 2015), leading to profound metabolic depression (Fu et al. 2017). These data further support the complexity and severity of MPS III manifestations in humans. Previous studies also showed broad metabolomic impairments in liver in mouse models of MPS I and MPS VII (Woloszynek et al. 2009), suggesting that metabolic abnormalities are common to MPS disorders, which is unsurprising given that these are closely related diseases and share many pathological features.

Importantly, we also demonstrate that the depressed amino acid metabolism in MPS III patients involves the majority (virtually all) of essential, conditionally essential, and non-essential amino acids, as observed in MPS IIIB mice (Supplementary Table S2) (Fu et al. 2017), indicating broad functional defects in amino acid metabolism, including absorption, transport, biosynthesis, and catabolism. Similar disturbances with reduced function also occur across the majority of critical metabolism pathways, involving lipids, carbohydrates, peptides,

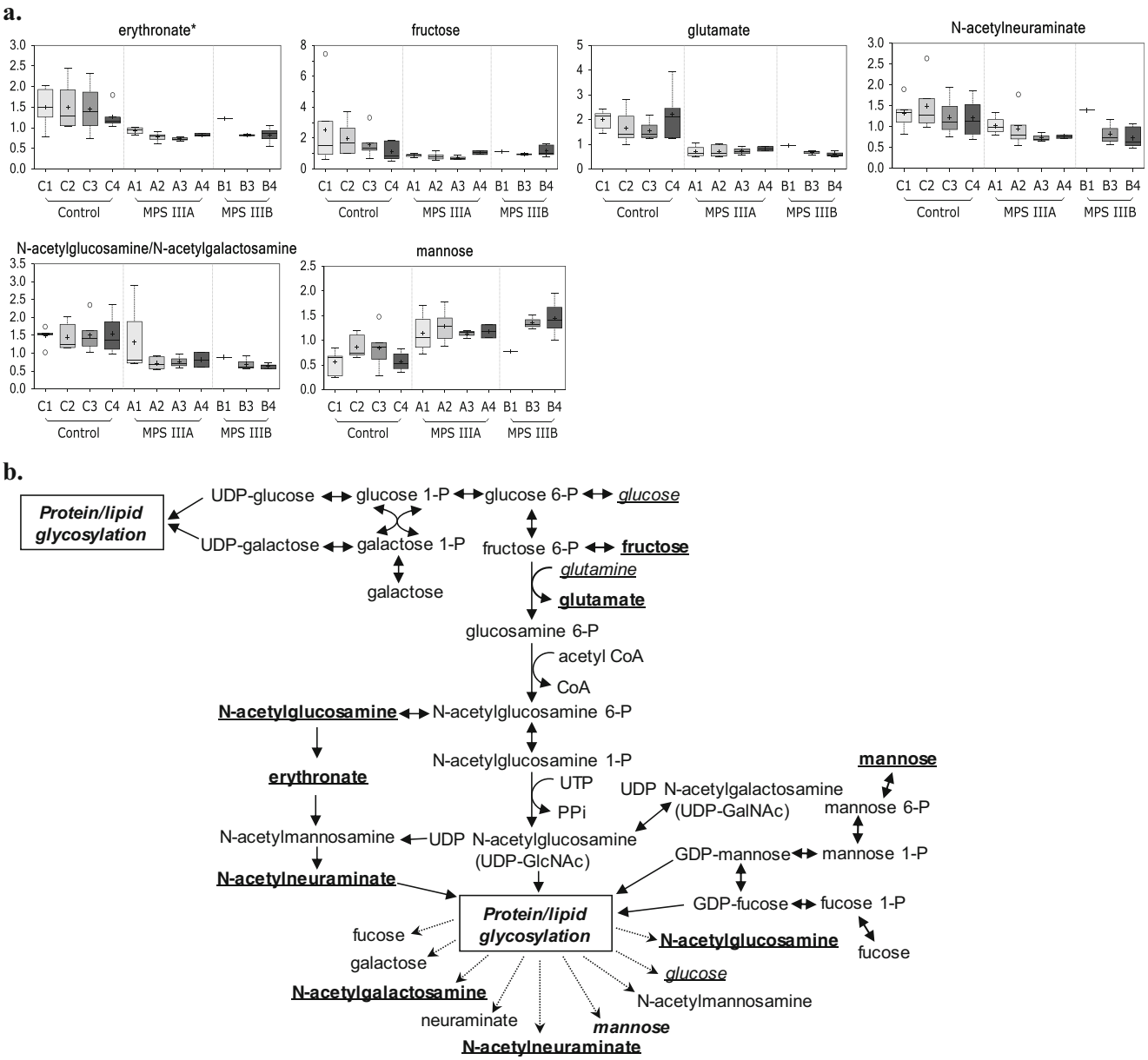


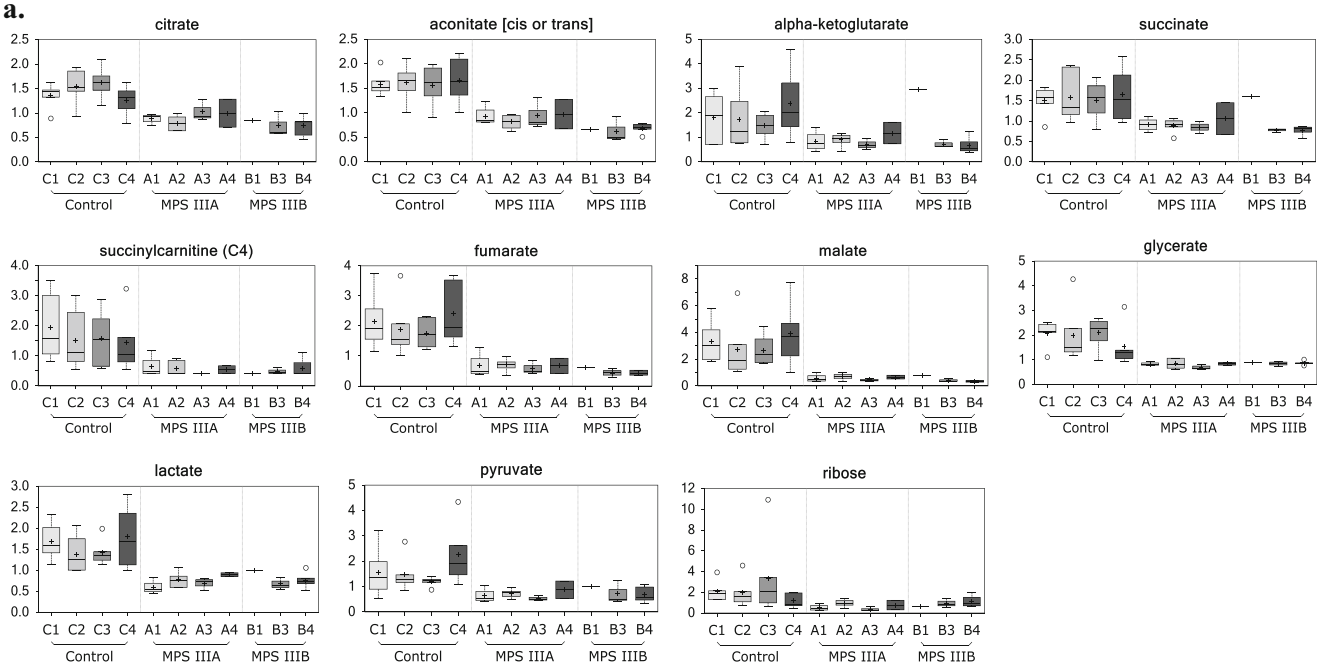
Fig. 4 Broad disturbance of glycosylation pathway in MPS IIIB during disease progress Serum samples from MPS III patients and healthy control subjects were analyzed by global metabolomics profiling using mass spectrometry. **a.** Metabolomic comparison: data presented as Scaled Intensity (**y-axis**) using box and whiskers plots, based on $p \leq 0.05$ MPS III vs. controls. **A1–4:** MPS IIIA (n = 15, age 2.1–9.2 yr); **B1–4;** MPS IIIB (N = 10, age 2.3–13.7 yr); **C1–4:** Healthy controls (n = 23, age 2-9 yr). **b.** Disturbance of serotonin and kynurenine pathway. **b.** Disturbance of glycosylation pathway: **Bold and underlined metabolites:** $p \leq 0.05$ MPS III vs. controls; **Underlined italic metabolites:** $p > 0.05$ MPS III vs. controls; **Others:** not included in the testing profile

nucleotides, and energy. This study may therefore provide important information for a deeper understanding of the disease mechanisms and therapeutic development for MPS III. Considering the breadth of metabolic impairment, treatment approaches such as managing diet are unlikely to be feasible or beneficial.

The significant reductions in key components of glycosylation pathways indicate the profound dysfunction of protein and lipid glycosylation. Glycosylation is critical for the function and stability of numerous proteins, lipids and other macromolecules (Varki et al. 2009). Therefore, the depressed state of glycosylation, as detected in MPS III patients in this study and in MPS IIIB mouse model (Supplementary Table S2) (Fu et al. 2017), may severely compromise the biological functions of macromolecules, further expanding the scope of metabolic impairments, and contributing to pathophysiological changes.

While in MPS III patients, the majority of altered serum metabolites were reduced, it is important to point out the detected elevation in derivatives of cysteine metabolism,

a.



b. Glycolysis and TCA pathway

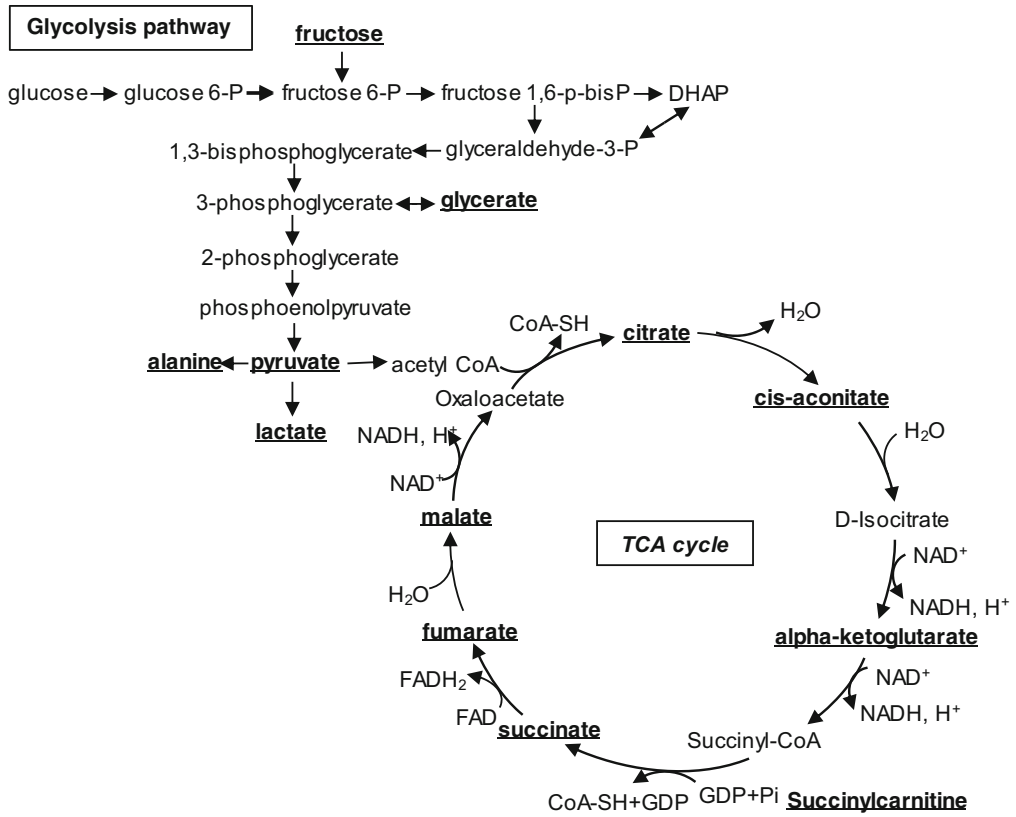


Fig. 5 Depressed energy metabolism in MPS III patients. Serum samples from MPS III patients and healthy controls were analyzed by global metabolomics profiling. **a.** Metabolomic comparison: data presented as Scaled Intensity (**y-axis**) using box and whiskers plots, based on $p \leq 0.05$ MPS III vs. controls. **A1–4:** MPS IIIA ($n = 15$, age 2.1–9.2 yr); **B1–4;** MPS IIIB ($N = 10$, age 2.3–13.7 yr); **C1–4:** Healthy controls ($n = 23$, age 2-9 yr). **b.** Disturbance of energy metabolism pathway: **Bold and underlined metabolites:** decrease in MPS III vs. controls $p \leq 0.05$; **Others:** not included in the testing profile

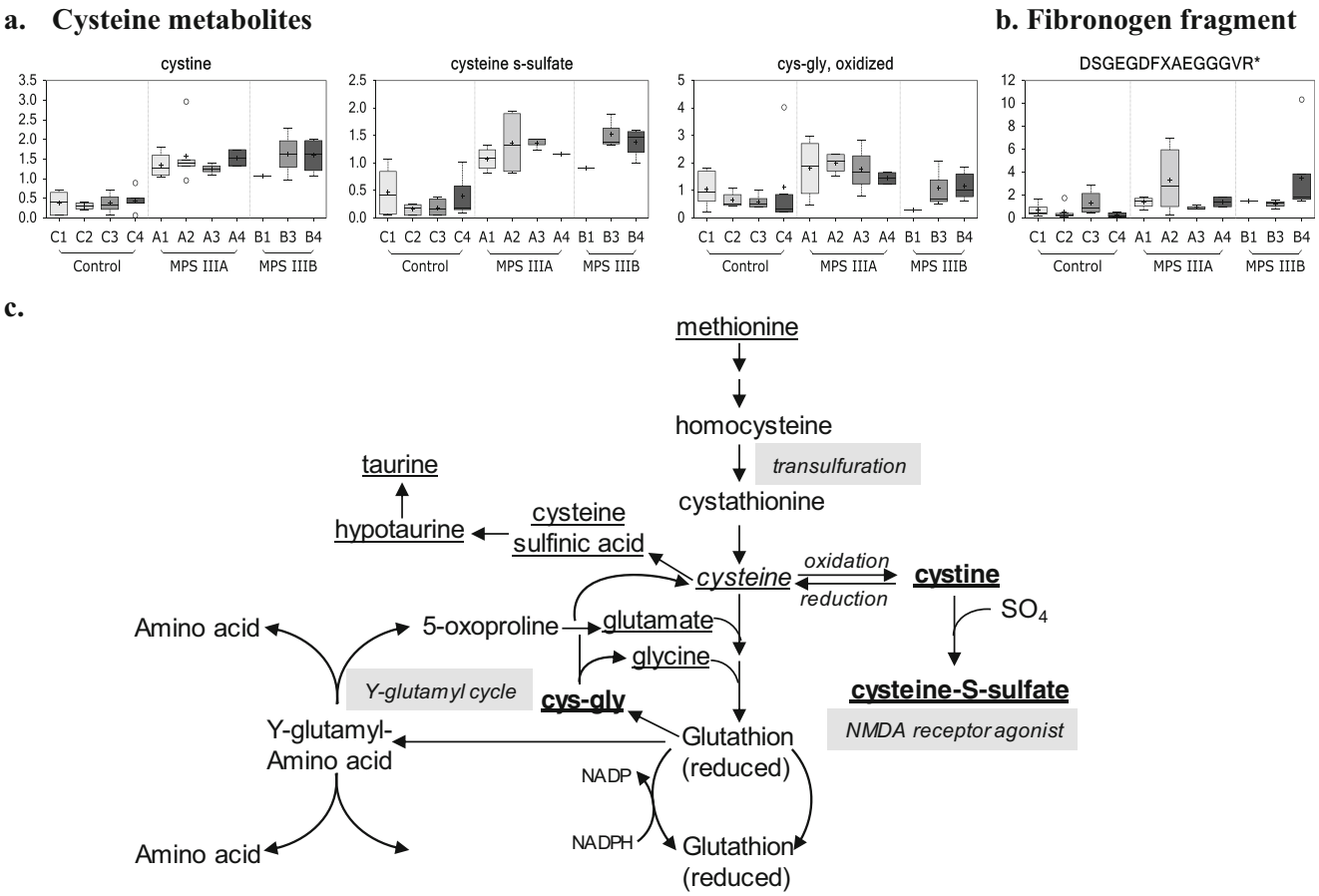


Fig. 6 Elevated cysteine metabolites and fibrinogen fragment in serum in MPS III patients Serum samples from MPS III patients and healthy control subjects were analyzed by global metabolomics profiling using mass spectrometry. **a, b.** Metabolomic comparison: data presented as Scaled Intensity (**y-axis**) using box and whiskers plots, based on $p \leq 0.05$ MPS III vs. controls. **A1–4:** MPS IIIA (n = 15, age 2.1–9.2 yr); **B1–4;** MPS IIIB (N = 10, age 2.3–13.7 yr); **C1–4:** Healthy controls (n = 23, age 2-9 yr). **c.** Disturbance cysteine metabolism pathway. **Bold and underlined metabolites:** increase $p \leq 0.05$ MPS III vs. controls; **Underlined italic metabolites:** $p > 0.05$ MPS III vs. controls; **underlined:** decrease $p \leq 0.05$ MPS III vs. controls; **Other metabolites and pathway components:** not included in the testing profile

fibrinogen α-chain peptide fragment and monoacylglycerols. The elevated cysteine metabolites may indicate oxidative stress, a known component of MPS III pathology (McGlynn et al. 2004; Villani et al. 2007; Villani et al. 2009), and may exacerbate the tissue damage via their cytotoxicity (Janaky et al. 2000). Fibrinogen is a glycoprotein that is essential in blood coagulation (Hoppe 2014), therefore the increase in fibrinogen peptide fragment may reflect elevated coagulation status, supported by previous findings of elevation in heparin cofactor II-thrombin complex (HCII-T) in MPS III patients (Langford-Smith et al. 2011). Further, while the mechanisms are unclear, the increases in nearly all measured monoacylglycerol metabolites indicate elevated lipase activity in MPS III patients, further highlighting the complexity of the pathology, given that elevations of serum lipase have been linked to numerous diseases and pathophysiological conditions (Hameed et al. 2015).

As observed in MPS IIIB mice (Supplementary Table S2) (Fu et al. 2017), one of the significant findings in this study is that the serum metabolomic profiles revealed severe depression of major neurotransmitter metabolism in MPS III in humans, involving pathways of glutamate, gamma-aminobutyric acid, aspartate, serotonin, and dopamine. In addition, there were significant reductions in multiple metabolites in the serum from MPS III patients, such as acetylcarnitine (ALC), N-acetylaspartate (NAA), 3-methoxytyrosine and taurine, which are linked to neurological functions and may play a role in neurological dysfunctions in MPS IIIB. Of them, acetylcarnitine (C2) possesses unique neuroprotective, neuromodulatory, and neurotrophic properties (Virmani and Binienda 2004). N-acetylaspartate (NAA) is a derivative of aspartate and the second most concentrated molecule in the brain, involved in neuronal osmosis, lipid and myelin synthesis in oligodendrocytes, synthesis of the important neurotransmitter N-acetylaspartylglutamate, and energy production in neuronal mitochondria (Moffett et al. 2007). Previous studies also showed that reduced NAA may play a role in motor neuron loss in ALS (Rothstein et al. 1990). 3-Methoxytyrosine is a major

extracellular metabolite of dopamine and a neuromodulator (Sotnikova et al. 2010). Taurine is the second most important inhibitory neurotransmitter in the brain following GABA, and low plasma taurine levels have been linked to variety of disease conditions (http://www.hmdb.ca/metabolites/HMDB00251). Further, the most reduced serum metabolite in MPS III patients was oleamide, which is an endogenous fatty acid amide inducing sleep in animals (Fedorova et al. 2001; McKinney and Cravatt 2005; Prospéro-Garcíaa et al. 2016). It has the ability to bind to the cannabinoid-1 receptor (CB1R) as a full agonist and may interact with multiple neurotransmitter systems (Prospéro-Garcíaa et al. 2016). It is unclear whether the reduced serum levels of these metabolites are contributors to, or consequences of, the severe neuropathy in MPS III. However, many neurotransmitters are synthesized from simple and abundant precursors, such as amino acids, which are readily available from dietary proteins broken down by digestion and transported via blood circulation to cells in the nervous system (Deutch and Roth 2003). It is therefore possible that the significant decreases in serum levels of neurotransmitters, neuro-associated compounds and derivatives are associated with the profound progressive MPS III neuropathic manifestations. Further, the detected dramatic reduction in serum oleamide may be linked to the sleep disorders that are typical in MPS III patients at the early to intermediate stages of disease progression.

Taken together, the vast assortment of metabolomic changes related to neurotransmitter function may provide the best candidate biomarker potential for reporting the CNS neuropathic status in MPS III patients. We previously demonstrated in MPS IIIB mice that the serum metabolomic profiles responded well to a single systemic delivery of the rAAV9-h*NAGLU* vector, a previously established effective gene therapy treatment targeting the root cause of the disease (Fu et al. 2011), leading to normalization of the majority (>87%), and over-correction of 13%, of serum metabolomic abnormalities (Fu et al. 2017). Notably, this rAAV9-h*NAGLU* gene delivery approach has recently been approved by the FDA for a Phase 1/2 clinical trial in MPS IIIB patients (IND# 16671, to be initiated), highlighting the urgent need for effective outcome reporters for CNS therapeutic effects. Given the resemblance of the MPS IIIB mouse model (Li et al. 1999) to the human disease(Neufeld and Muenzer 2001), we expect that metabolomics markers will be similarly resolved with effective treatment in patients.

Notably, this study demonstrate that the serum global metabolic profiles in MPS III patients are largely similar to those in MPS IIIB mice (Supplementary Table S2) (Fu et al. 2017). However, disparities were observed in some metabolites between MPS III patients and MPS mice, predominantly in lipid metabolites, including long chain fatty acids, polyunsaturated fatty acids and monoacylglycerol. The functional significance of these differences remains unclear and needs further investigation.

In summary, we demonstrate here that lysosomal accumulation of HS-GAGs triggers broad profound metabolic impairments in patients with MPS IIIA and IIIB, with predominantly reduced function in virtually all metabolism pathways, possibly involving absorption, transport and biosynthesis. Importantly, serum global metabolomics profiles are highly translatable between mice and humans with MPS III. Notably, we have recently demonstrated that the metabolic impairments responded well to an effective gene therapy approach in MPS IIIB mice, supporting the surrogate biomarker potential of serum metabolomic profiles (Fu et al. 2017).

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