**METABOLIC CHARACTERIZATION OF NEURODEVELOPMENTAL DISORDERS WITH GLUTAMATERIGC NEUROTRANSMISSION AFFECTATIONS.**

# ABSTRACT

The study of neurodevelopmental disorders (NDDs) has been challenging due to the heterogeneity of their genetic etiology and their complex pathophysiology. The use of a metabolomic approach for the study of these pathologies can provide both insight into their pathophysiology and outline novel therapeutic alternatives.

We performed a targeted metabolomics analysis on CSF samples from 37 patients with alterations in synaptic metabolism and 5 controls. Samples from patients with Rett syndrome (RTT), GRINpathies or STXBP1 encephalopathy were included. We performed univariate (UVA) and multivariate statistical analysis (MVA), using Wilcoxon rank-sum test, principal component analysis (PCA), and oPLS-DA. By using the results of both analyses, we identified the biomolecules that were significantly altered and that were important in the separation of the respective groups. On these, we performed pathway- and network-based analyses to define which metabolic pathways were possibly altered in each pathology.

In Rett syndrome samples, we observed alterations in the phenylalanine, tyrosine and tryptophan metabolism pathways, which interestingly depend on the same transporter to cross the blood-brain barrier (BBB). Analysis of the expression of LAT1 transporter in brain samples from a mouse model of Rett syndrome revealed a decrease in the transporter expression, that was already noticeable at pre-symptomatic stages.

Studying neurodevelopmental disorders from this perspective advances the understanding of their pathophysiology, shining light on an under-studied feature as is their metabolic component.

# INTRODUCTION

Neurodevelopmental disorders (NDDs) are a group of pathologies that affect normal brain development, including a plethora of diseases with different clinical presentations, ranging from attention deficit or autism spectrum disorders (ASDs) to other monogenic diseases as Rett or Fragile X syndromes (REF).

The pathophysiological landscape and genetic causes of neurodevelopmental diseases are diverse. Interestingly, many of the genes responsible for NDDs are involved in pathways related to protein synthesis, transcriptional regulation, and synaptic signaling (reviewed in Mullins et al. 2016). Because of this, there has been great interest in the biomolecules involved in these pathways as potential biomarkers or therapeutic targets for different NDDs. For example, several studies have found the excitatory neurotransmitter glutamate to be increased in the blood and brain tissue of ASD patients, leading to the hypothesis that some types of ASD is caused by an increase in the excitation/inhibition (E/I) ratio due to either a lack of GABA-ergic neurons or a deficiency in their activity (Rubenstein and Merzenich, 2003; Nelson and Valakh, 2015; Robertson et al., 2016; Uzunova et al., 2016; Lopatina et al. 2019). Similarly, Rett syndrome (RTT) patients and animal models have shown higher levels of glutamate and a reduced expression of metabotropic glutamate receptors 5 and 7 (mGlu5, mGlu7)  (Gogliotti et al. 2016; Gogliotti et al. 2017), together with a clear involvement of the GABAergic system.

Besides defective glutamatergic neurotransmission, other elements contribute to their evolution and clinical presentation, such as inflammation, neuronal development of metabolism.  Their study will advance in the development of new biomarkers for neurodevelopmental diseases along with therapeutic strategies that can address shared aspects of their pathophysiology.

Omic technologies are emerging as useful approaches to complex diseases, advancing in their description and identification of biomarkers. Metabolomics is a relatively new area of research that can capture patient-specific variation such as response to treatment, exposure to environmental conditions, or disease progression, which makes it a promising avenue for personalized medicine. One of the main advantages of metabolomics is that it offers a more accurate picture of the phenotype. It allows researchers to study not only the information encoded in the genome of a patient, but also the effect that genomic, proteomic regulation have on its expression. For diseases like NDDs, the metabolomic approach will provide the missing link between their molecular profiles and their clinical presentation.

In this work we have studied the metabolic profile of patients affected with different NDDs in which glutamatergic neurotransmission had been proposed as a major event in the disease progression, comparing patients CSF samples with healthy controls. The joint study of different pathologies that display common molecular features has helped on the identification of shared pathophysiological mechanisms, common to neurodevelopmental diseases.

# MATERIALS AND METHODS

**PATIENTS AND SAMPLES**

Cerebrospinal fluid (CSF) was collected from a total of 25 patients and 5 healthy controls. Patients presented mutations in the genes *CDKL5* (n=2), *GRIN* (n=7), *MECP2* (n=12), and *STXBP1* (n=4). The median age at the time of collection was 6 years, ranging from infancy to 18 years. The majority of patients were female (n=19) due to the large proportion of Rett syndrome patients that were used in this study.

**METABOLOMICS ANALYSIS:**

Samples were sent to the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, where a targeted metabolomics analysis was conducted by liquid chromatography with tandem mass spectrometry (LC-MS-MS) for tryptophan related metabolites and acylcarnitines. Gas chromatography–mass spectrometry (GC-MS) was performed for organic acids and sugar metabolites related to energy metabolism and the TCA cycle. A total of 82 metabolites were detected, and the results were presented as micromolar (μM) concentrations.

**LC-MS/MS:** CSF (50 μL) samples were diluted in methanol (200 μL, 100%) and the set of labelled internal standards (DL-Kynurenine, DL-Tryptophan, 5-Hydroxyindole-3-acetic acid, L-Citrulline, 2-Picolinic acid, Nicotinic acid, Kynurenic acid, Indole-3-acetic acid (Sigma Aldrich); Serotonin hydrochloride, rac Kynurenine-d4 Trifluoroacetic Acid Salt, D-Tryptophan-d5, 5-Hydroxyindole-3-Acetic Acid-d2 (5-HIAA-d2), Serotonin-d4 Hydrochloride, 2-Picolinic-d4 Acid, Nicotinic acid-d4, 3-Hydroxyanthranilic acid, 3-Hydroxyanthranilic acid-d3, Kynurenic acid-d5, Indole-2,4,5,6,7-d5-3-acetic-α,α-d2 Acid, 3-Indolepropionic-d2 Acid, N-Acetyl-5-hydroxytryptamine (Toronto Research Chemicals); Trigonelline Hydrochloride (TCI); Anthranilic acid (Glentham life sciences); Hydrocortisone, Androstenedione, Testosterone, Progesterone (Sigma Aldrich and Toronto Research Chemicals). Following centrifugation (10 min, 15000 rpm, 4ºC), the supernatant was dried in a SpeedVac concentrator and reconstituted in methanol (50 μL, 100%).

The equipment consisted on an UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series (Agilent Technologies) and a Kinetex 2.6 μm Polar C18, 100 Å, 150 x 2.1 mm (Phenomenex) analytical column. Chromatographic separation was performed in mode negative electrospray ionization (ESI) at 20ºC with an injection volume of 1 μL. Mobile phase A was formic acid (0.1%) and mobile phase B was methanol:formic acid (10:1, v/v).

**GC-MS:** CSF (50 μL) samples were diluted in water:methanol (200 μL, 8:2, v/v) and the set of labelled internal standards (Succinic-d4 acid, myristic-d27 acid, d-glucose 13C6 and L-Methionine-(carboxy-13C,methyl-d3) (Sigma Aldrich)). Following centrifugation (5 min, 15000 rpm, 4ºC), the supernatants were dried in a SpeedVac concentrator at 45ºC and reconstituted in methoxyamine (30 μL, 100%). Samples were incubated at 37ºC for 90 min. and then silylated with 45 μL of MSTFA + 1 % at room temperature for 60 min.

The separation was performed on a GC-QTOF 7200 and a HP5-MS UI capillary column (30 m x 250 μm I.D., 0.25 μm film thickness), both from Agilent Technologies. Helium (>99.999%) was used as the carrier gas with a constant flow 1.1 mL/min. Initial oven temperature was set at 60ºC, then increased by 10ºC/min. to 320ºC and held constant for 10 min. Samples were injected in split mode 1:20 at injection temperature 250ºC. Compounds were detected through MS in electron ionization (70 eV) and full-scan monitoring mode (m/z 50–600) mode with an acquisition rate of 5 spectra/s. Ion source temperature was 250ºC and quadrupole temperature was 200ºC.

Organic acids were identified and semi-quantified using the spectra library Fiehn-pct-2013 and their pure analytical standards (Pyruvic acid, lactic acid, glycolic acid, 3-hydroxybutyric acid, glycerol, succinic acid, glyceric acid, fumaric acid, malic acid, d-threitol, threonic acid, α-ketoglutaric acid, arabitol, glycerol-1-phosphate, 3-phosphoglyceric acid, citric acid, d-mannitol, myo-inositol, glucose-6-phosphate, d-sucrose and α-tocopherol (Sigma Aldrich)).

**STATISTICAL ANALYSIS**

The goal of the analysis was to compare the metabolic profiles of patients to those of healthy controls. All statistical tests were conducted in R version 4.3.0. Metabolic profiles of patients were compared to those of health controls, either all together or categorized on the glutamatergic behavior of the disease (hypo-glutamatergic diseases vs hyper-glutamatergic + GABAergic).

The metabolite concentrations were log2-transformed in order to account for the expected high variability and noise found in metabolomics data. Multivariate statistical methods were applied on the transformed data in order to account for the small sample size and the inherent multicollinearity of the data. Unsupervised principal component analysis (PCA) was performed in order to obtain an overall view of the variation between samples and their separation into clusters. Ward hierarchical clustering with Euclidian distance combined with a heatmap (pheatmap version 1.0.12) was used to compare the log-transformed concentrations of all the analyzed metabolites in patients and controls. Orthogonal partial least-squares discriminant analysis (OPLS-DA) from the ropls package (version 1.32.0) was used as a supervised model to identify metabolites responsible for group separation. Models were evaluated by goodness of fit (R2Y), goodness of prediction (Q2Y), and the root mean square error of estimation (RMSEE). 7-fold cross-validation was performed to mitigate the risk of overfitting caused by the high dimensionality of the data. Metabolites were selected based on their variable importance in projection (VIP) scores (VIP > 1). Permutation testing (n=1000) was used to determine the statistical significance of the results (p<0.05).

The results of the classification models were corroborated by performing Wilcoxon rank-sum tests comparing each metabolite concentration in patients and in controls. The non-parametric test was chosen due to the small number of samples. Correction for the false discovery rate (FDR) was applied in order to account for the issue of multiple testing (p<0.05).

Pathway analysis was performed for metabolites with p<0.05 or VIP score > 1 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the KEGGREST package (version 1.40.0). Pathways were identified as important when two or more of their metabolite components were found to be altered between the disease groups and the healthy controls. The results were plotted after excluding pathways that are ubiquitous or unrelated to synaptic metabolism. Special attention was given to the tryptophan metabolism pathway, as well as to the amino acid substrates of Large Neutral Amino Acid Transporter (LAT1).

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Proteins were extracted from brain isolates through a 30’ cold incubation with RIPA and protease inhibitors. Following extraction, proteins were quantified by Bradford method and prepared at a homogenous concentration in Laemmli Buffer in reductive denaturing conditions and subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with TBS-Tween (0.05%): milk 5% for 1h at room temperature. Primary antibodies were incubated O/N at 4ºC in blocking buffer, at the following concentrations: SLC CONCENTRATION. Tubulin (1:20,000, sc-59803, Santa Cruz Biotechnology) was used as a loading control. The secondary antibodies used were HRP-conjugated goat anti-Rabbit and goat anti-Mouse IgG antibodies (Thermo Fisher) and were detected using the PierceTM ECL Western Blotting substrate (Thermo Fisher). Quantification of protein expression was performed using Fiji software.

Both B6.129P2(C)-*Mecp2tm1.1Bird*/J and control littermates mice were used. To increase the translational value of the work, only female mice have been used, as they have been reported to better recapitulate the disease phenotype. Mice were housed in standard cages with ad libitum access to food and water and in controlled environmental conditions of light (12 h dark/light cycle starting at 7:30 am), temperature (22°C) and humidity (60%). Mice were genotyped following the provider recommendations.

# RESULTS

Cerebrospinal fluid (CSF) samples were collected from 29 individuals with diseases affecting glutamate/GABAergic balance: 14 Rett patients (12 bearing mutations in MECP2 and 4 in CDKL5), 10 hypo-glutamatergic patients (6 GRIN2B patients and 4 STXP1 patients) and 5 controls. All the patients had known mutations affecting the respective genes (Supplementary Table 1). All patients and controls were within pediatric age, ranging from 2 to 15 years old. Patients and controls from both genders were included, though the majority were female because of the higher prevalence of RTT on girls (Figure 1A).

All CSF samples were subjected to a semi-targeted metabolomic analysis, through which we measured the concentrations of metabolites related with energy and amino acids metabolism, with a special coverage of tryptophan and tyrosine metabolism. The total concentration of metabolites detected was similar among all samples (Supplementary Figure 1), giving confidence in the results and in the differences observed in each population. The analysis identified a total of 68 metabolites (Supplementary Table 2). Dimensionality reduction through Principal Component Analysis (PCA)  revealed that, despite some crossovers, patients from both groups were more similar to each other than to the controls, as they assembled in two different clusters (Figure 1B). This profile was maintained after separating  RTT and hypo-glutamatergic patients (Figure 1C,D), where again, both CDKL5 and MeCP2 patients, as well as STXBP1 and GRIN2B patients were more similar to each other than to controls. Hierarchical clustering with Ward’s method criterion a clustering algorithm that  finds groups with the least internal variance, was performed using all the analyzed metabolites. This revealed that both groups had a similar underlying structure, with two main clusters contaning metabolic alterations consistent across these different pathologies.

In order to overcome the overlapping of controls and patients and to prioritize clinically relevant metabolic differences we performed feature selection through the  supervised method Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). This technique efficiently differentiated patients from controls in both scenarios (RTT and hypo-glutamatergic samples) (Figure 2A, B).  The models showed a strong ability to discriminate between classes (R2Y=0.939and 0.960 for RTT and hypo-glutamatergic respectively), and a good prediction accuracy predictive accuracy ≥ 0.8 (Q2Y= 0.816 and 0.866) after permutation (n=1000). The models identified 25 key metabolites in the RTT group and 17 in the hypo-glutamatergic one, 12 of which were common within both groups, revealing a possible that shared metabolic pathways contribute to their pathophysiology (Figure 2C-D). To determine which pathways were most impacted by the alterations in metabolite concentrations, we assessed KEGG pathway over-representation for metabolites that had  a *p* < 0.5 level or VIP score >1. The most affected pathways were related to amino acid metabolism and transport, in particular tryptophan and its metabolites. (Figure 2E)

In humans, tryptophan (Trp) metabolism can occur through two different pathways: the kynurenine pathway, through which Trp is degraded into kynurenic acid and 2-picolinic acid, and the serotonin pathway, converting Trp into 5-HT for final production of serotonin, metabolized into 5-hydroxyindoleacetic acid (5-HIAA.). Several of these metabolites were found to be decreased in patients, although tryptophan itself was not. As shown in figure 3, even though there were no statistically significant differences in the concentration of tryptophan, we detected a tendency to decrease in the concentration of metabolites of both pathways. Samples from both groups showed a decrease in both kynurenine (RTT: 0.009 uM, hypo-glutamatergic: 0.008 uM, controls: 0.079 uM) and kynurenic acid ( RTT: 0.0004 uM, hypo-glutamatergic: 0.0002 uM, controls: 0.004 uM), though only the RTT decrease in kynurenine was statistically significant. Moreover, both groups had significantly decreased concentrations of 5-hydroxy-3-indoleacetic acid (RTT: 0.109 uM, hypo-glutamatergic: 0.077 uM, controls: 0.436 uM).

Interestingly, tryptophan and its metabolic derivatives were not the only ones with reduced concentrations in patients.  Both groups showed decreased concentrations of two of the three branched-chain amino acids (BCAAs) (Val: RTT: 51.154 uM, hypo-glutamatergic: 60.427 uM, controls: 65.424 uM;  Leu: RTT: 28.775 uM, hypo-glutamatergic: 34.484 uM, controls: 45.910 uM; Ile: RTT: 8.409 uM, hypo-glutamatergic: 8.582 uM, controls: 9.461 uM) . Similarly, phenylalanine was found to be significantly decreased in both groups (RTT: 23.920 uM, hypo-glutamatergic: 27.016 uM, controls: 89.806 uM), while the tyrosine metabolite  4-hydroxyphenyllactic acid was only reduced in RTT patients (RTT: 1.453 uM, hypo-glutamatergic: 2.811 uM, controls: 2.470 uM)  In contrast, all patients showed threonine concentration values within a control-range (RTT: 114.431 uM, hypo-glutamatergic: 148.299 uM, controls: 137.977 uM) (Figure 4A).

Valine, leucine, isoleucine, threonine, tryptophan, tyrosine, and phenylalanine, along with other large neutral amino acids, are preferentially transported across the blood-brain barrier through a facilitative Na+-independent transporter named LAT1 (system L Amino Acid transporter), coded by the *SLC7A5* gene. We investigated its expression in a model of RTT, the BIRD female mice. Remarkably, brain samples from RTT female mice showed a significant decrease in the expression of LAT1, compared to littermate controls (Figure 4B). This was observed at two different neurodevelopmental stages, 3 and 7 months old mice, pointing towards a potential involvement of amino acids brain transport and metabolism in the pathophysiology of several neurodevelopmental diseases.

Integrating the results of the multivariate and univariate analyses showed that both disease groups appear to have altered concentrations of the same metabolites, even though RTT patients had more statistically significant differences (Figure 5A-B). Performing hierarchical clustering on only the selected metabolites resulted in perfect separation of patients and controls (Figure 5C-D), which further showed that only trigonelline and indole-3-propionic acid had increased concentrations in both groups of patients, while 3-hydroxyanthanillic acid was only increased in RTT patients.

Although not profoundly studied in this paper, other metabolic routes appeared to be involved in the diseases’ pathophysiology. Besides amino acids metabolism, aminoacyl tRNA biosynthesis metabolism appeared over-represented. Other non- amino acids-related pathways included pentose phosphate pathway and TCA cycle, exemplifying the importance of energy and oxidative stress metabolism transversal to neurodevelopmental diseases. Finally, metabolites such as indole-3-acetic and indole-3-propionic acids pointed towards a possible involvement of   the gut microbiome and the gut-brain axis in neurodevelopmental disorders.

# DISCUSSION

Defective glutamate and GABAergic neurotransmission is one of the main features outstanding in the pathophysiology of neurodevelopmental diseases. They range from loss-of-function mutations in *GRIN2B* that impair the response of NMDA receptors to glutamate neurotransmission (García-Recio et al. 2021; Wang et al. 2022) to the unbalance between GABA and glutamate neurotransmission that is characteristic in RTT (Meng et al. 2016; Ip et al. 2018). However, other elements also contribute to the landscape of neurodevelopmental diseases, such as inflammation (Dunn et al. 2019; Yan et al. 2023), neuronal maturation (Zengeler et al. 2021), and neuronal metabolism (Lee et al. 2013). Specifically, metabolic alterations are known to play key roles in the pathophysiology of neurodevelopmental diseases, and they have been explored in several non-metabolic diseases, such as RTT (Neul et al. 2020), autism spectrum disorder (ASD) (Graham et al. 2020), psychiatric disorders (Reviewed in Penninx & Lange, 2022), and epileptic encephalopathies (Reviewed in Rho et al. 2022). The study of metabolic alterations in neurodevelopmental pathologies could advance the knowledge of the etiology of these diseases, as well as provide potential biomarkers and new therapeutic targets.

In our work we have studied the neurometabolic component of four neurodevelopmental diseases in which the main element of their pathophysiology is an altered glutamatergic neurotransmission. Specifically, we have analyzed cerebrospinal fluid samples from 30 patients (with mutations in *STXBP1, GRIN2B, MECP2* and *CDKL5*) compared to controls, focusing on the study of amino acid metabolism. Studying these alterations in CSF samples adds complexity to the study, especially due to the difficulty of obtaining samples from controls, but allows us to understand more accurately what is occurring in the brains of patients during the course of the disease. One of the main questions raised by this work is to understand whether the metabolic alterations associated with defects in glutamatergic neurotransmission may be common to several pathologies, or if each disorder has unique metabolic profile. Our study supports the notion that there is a shared metabolic component to all the diseases included, which provides a possible avenue for therapies with broad phenotypic applicability.

Analysis of metabolic alterations in patients and controls CSF has revealed common alterations shaping the pathophysiology of neurodevelopmental diseases. Multivariate feature selection identified 18 influential metabolites for RTT patients and 17 for hypo-glutamatergic disorders, while univariate hypothesis testing found 17 significantly altered metabolites in RTT and 7 in hypo-glutamatergic disorders. There were 10 metabolites in RTT and 7 in hypo-glutamatergic disorders that were selected by both methods. Of these, only 4 metabolites (3-hydroxyanthranilic acid, trigonelline, galactose, and indole-3-propionic acid) had increased concentrations in RTT and 2 (trigonelline and indole-3-propionic acid) in hypo-glutamatergic disorders.

While previous work on the study of metabolic alterations in neurodevelopmental diseases has focused on pathways as energy metabolism, redox homeostasis or lipid metabolism, the role of amino acids metabolism in non-metabolic diseases has been understudied. This is significant because amino acids are essential players in brain function and development: not only as necessary components of protein synthesis, but also as neuromodulators and biosynthetic precursors of various neurotransmitters. Primary dysregulation of their metabolism results in neurodevelopmental pathologies such as aromatic l-amino acid decarboxylase (AADC) deficiency (Wen et al. 2020) (impaired tyrosine and tryptophan metabolism); the pediatric parkinsonism tyrosine hydroxylase deficiency (THD) (impaired conversion of tyrosine to dopamine) (Dong et al. 2020); Maple Syrup Urine Disease (MSUD) (Blackburn et al. 2017) and a treatable form of autism (BCAA metabolism) (Tangeraas et al. 2023). These pathologies exemplify the importance of amino acid metabolism in the function of the developing brain. Their role in the regulation of neurodevelopment and neurotransmission make it important to study these pathways in pathologies that are not traditionally linked to amino acid metabolism itself.

Pathway analysis of our data showed that these metabolites were mostly involved in amino acid and energetic metabolism, especially in tryptophan and galactose metabolism. The two groups of patients showed highly similar metabolic profiles, though 11 metabolites (3-hydroxyanthranilic acid, leucine, glycerol, d-xylitol, 4-hydroxyphenyllactic acid, d-fructose, galactose, d-gluconic acid, saccharic acid, myo-inositol, sedoheptulose) were identified as altered on RTT but not on hypo-glutamatergic disorders. This could be due to the smaller number of hypo-glutamatergic patients that were available for the study, and it may be worthwhile for further studies to replicate this analysis on a larger cohort of these specific disorders.

One of the pathways were we found alterations common to all the diseases is tryptophan metabolism. Tryptophan is an essential amino acid that is the precursor of several neuroactive compounds. The majority of tryptophan is catabolized through the kynurenine pathway into the coenzyme nicotinamide adenine dinucleotide (NAD+), which is a vital component of energy metabolism. Kynurenic (KYNA) is an intermediate metabolite of the kynurenine pathway that acts as an antagonist to the N-methyl-D-aspartate receptors (NMDARs). On the other hand, tryptophan can be metabolized through the serotonin pathway, by which the neurotransmitter serotonin is synthesized. We have found a decrease in the concentration of different metabolites in both metabolic branches, as samples from all disease groups showed a reduction in both kynurenine and kynurenic acid concentrations, together with lowered concentrations of 5-hydroxy-3-indoleacetic acid. Alterations in the kynurenine pathway have been reported in neurological conditions as diverse as migraine (Fila et al. 2021), schizophrenia (Holtze et al. 2012), epilepsy (Żarnowska et al. 2019), and ASD (Lim et al. 2016; Savino et al. 2020). Yet, it is noteworthy that such alterations usually refer to increases in the kynurenine pathway. Ptchd1 KO mice showed marked increases in  brain kynurenine pathway metabolite concentrations, associating an activation on the KP with ADHD-like behavior. Similar to that, increases in the kynurenine pathway have been associated to schizophrenia and bipolar disorder (Erhardt et al. 2017), and kynurenic acid has been reported to be significantly increased in the cerebrospinal fluid (CSF) and brain tissue of patients with schizophrenia (Sathyasaikumar et al. 2011; Holtze et al. 2012).

Alterations in tryptophan metabolism are often associated with alterations in other amino acids (Fernstorm, 2013). Specific analysis of the concentration of large neutral amino acids in our samples has revealed a generalized decrease in all the diseases. A previous work on ASD surprisingly reported that alterations in the concentration of BCAAs were negatively correlated with the concentrations of glutamine, glycine, and ornithine in ASD patients but not in neurotypical controls (Smith et al, 2019). Their study highlighted the fact that the ASD population they studied was very heterogeneous, and the alteration they found was only able to identify a subset of 16.7% (86 out of 516 ASD patients) of ASD patients with very high accuracy (>90%). We did not find the same to be true for our patients, but saw rather that most of these amino acids were decreased, though not significantly, in all the diseases studied. In addition, their protocol specifically excluded syndromic patients in order to focus exclusively on ASD. This could either mean that a larger cohort of patients with the diseases included in this study could yield a group with  similar imbalances in amino acid concentration, or that non-syndromic ASD patients have different metabolic profiles from patients with syndromes that have autistic symptoms.

Interestingly, a metabolomic analysis of *Mecp2-/y* mouse cortex reported tryptophan and other amino acids (alanine, leucine, glutamate, isoleucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine) to be significantly increased when compared to controls, while only lysine was decreased (Golubiani et al, 2021). Although these changes were not observed on full brain extracts, where only increases in glutamine and non-amino acid metabolites were observed (Viola et al, 2007). These paradoxical results could be due to the alterations in amino acid concentrations being dependent on the brain region studied (El-Khoury et al. 2014).

Tryptophan is transported into the brain through the Large Neutral Amino Acids Transporter (*LAT1*, coded by the SLC7A5 gene). This protein is also responsible for the transport of other amino acids such as BCAAs, Threonine or Phenylalanine, which was reduced in our patients’ CSF. We have analyzed the transporter expression in brain tissue from a mouse model of RTT, noticing a decrease that was already detectable at pre-symptomatic stages. We find this decrease specially relevant, as it has been reported that SLC7A5regulates Kv1.2 potassium channels, modifying the functional outcomes of epilepsy-linked channelopathies (REF PMID: 30356053), and mutations SLC7A5 (together with other variants in genes encoding for Large Amino Acid Transporters) increase the risk of Autism Spectrum Disorder (REF PMID: 31701662).

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Metabolism can be a common feature in neurodevelopmental diseases worth studying.

How can these alterations be used for therapeutic purposes, or worth studying as we have identified them in ALL the diseases. Tryptohan supplementation?

Kynurenic acid is a NMDA receptor antagonist. KYNA has a possible neuroprotective effect: it is able to mitigate the neuronal loss in excitotoxic, ischemia-induced and neuronal injuries (Smith et al. [1993](https://link-springer-com.sire.ub.edu/article/10.1007/s00702-011-0665-y#ref-CR139); Gigler et al. [2007](https://link-springer-com.sire.ub.edu/article/10.1007/s00702-011-0665-y#ref-CR39)).  Could this reduction have an impact in the excitotoxicity associated with glutamatergic alterations?

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# FIGURE LEGENDS

***Figure 1. Unsupervised multivariate analysis of patient and control CSF metabolite concentrations. A) Demographic characteristics. B- D. PCA score plots showing the separation of patients from controls on the first two principal components.*** *B) All samples**C) MeCP2 and CDKL5. D) hypo-glutamatergic.* ***E and F. Ward hierarchical clustering showing log-transformed metabolite concentrations.*** *Both MeCp2 /CDKL5 (E) and hypo-glutamatergic (F) patients form homogeneous groups regardless of genotype. There is some overlap with controls, but mostly patients cluster together and have a markedly decreased expression of most metabolites*

**Figure 2. Identification of significantly altered metabolites and their impact on group classification A-B)** OPLS-DA score plots showing separations of controls compared to **A) RTT** and **B) hypo-glutamatergic patients**. Although control samples showed high intergroup variability, both OPLS-DA models showed good separation between the groups (R2Y(cum) > 0.9 ,Q2Y(cum) > 0.8, RMSEE < 0.2). C-D) VIP scores and corresponding p-values. Both diseases show similar metabolic alterations, though RTT syndrome patients (C) had a higher number of significantly altered metabolites. The highest VIP scores in both cases belonged to tryptophan metabolites. **E) Pathways affected by selected metabolites**. Altered metabolites for both RTT and hypo-glutamatergic patients had the highest impact in galactose metabolism, amino sugar and nucleotide sugar metabolism, and glycerolipid metabolism. The pathways that were most altered were ABC transporters, galactose metabolism, and tryptophan metabolism.

**Figure 3. Alterations in tryptophan metabolism**. Of the metabolites involved in tryptophan metabolism that were analyzed, only 5-Hydroxyindole-3-acetic acid was significantly decreased in both disease groups, while kynurenine was significantly decreased in RTT patients and non-significantly decreased in hypo-glutamatergic patients. Tryptophan, N-Acetyl-5-hydroxytryptamine, anthranilic acid, and kynurenic acid showed a slight decrease that was not statistically significant in both RTT and hypo-glutamatergic patients. 3-Hydroxyanthranilic acid did not vary between either group and the controls.

**Figure 4. Alterations in the metabolism of other amino acids. A)** All patients showed decreased levels of amino acids transported by LAT1. Leucine, phenylalanine, and the tyrosine metabolite 4-hydroxyphenyllactic acid were significantly decreased in RTT patients, while hypo-glutamatergic patients showed a similar tendency but were only significantly altered in phenylalanine and had slightly increased 4-hydroxyphenyllactic acid. Both groups had non-significantly decreased levels of valine.

**Figure 5. Results of multivariate and univariate statistical analyses A-B) total number of altered metabolites A) RTT B) hypo-glutamatergic patients**. For both groups there was a small number of metabolites identified as altered by both univariate and multivariate analyses. Both has metabolites with VIP scores > 1 that were not significantly altered, but only RTT had significantly altered metabolites with VIP scores < 1. This may be due to the larger sample size available for RTT patients. **C-D)** Both diseases show similar metabolic alterations, though RTT (C) had a higher number of significantly altered metabolites. Interestingly, RTT patients showed some metabolites that were significantly altered but did not contribute to the classification of the OPLS-DA model.