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

# 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.

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| Age, years, median (range) | 6 (0-18) |
| Sex, female, n (%) | 19 (76) |
| Intellectual disability, n (%) | 19 (76) |
| Encephalopathy, n (%) | 17 (68) |
| Epilepsy, n (%) | 18 (72) |
| Autism, n (%) | 3 (12) |

**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 (Supplementary information). 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 (Supplementary information). 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 an 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 using the spectra library Fiehn-pct-2013. Ion source temperature was 250ºC and quadrupole temperature was 200ºC. The acquisition rate was 5 spectra/s.

**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 Mann–Whitney 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).

# 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.

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 1A). This profile was maintained after separating RTT and hypo-glutamatergic patients (Figure 1B,C), 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 conta metabolic alterations are 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, from loss-of-function mutations in *GRIN2B* that result in the inability of NMDA receptors to respond to glutamate neurotransmission to the unbalanced between GABA and glutamate characterized in Rett syndrome. Yet, other elements contribute to the landscape of neurodevelopmental diseases, such as inflammation, neuronal maturation or metabolism. Specifically, metabolic alterations are known to play key roles in the pathophysiology of neurodevelopmental diseases. They are EXPLAIN TWO EXAMPLES. In our work, we have explored the metabolic profile of almost 30 patients with different neurodevelopmental diseases in which the metabolic component of the pathophysiology was understudied compared to the glutamatergic neurotransmission alterations.

Systemic analysis of metabolic alterations in patients and controls CSF has revealed common pathophysiologyical pathwas

SUMMARIZE OUR RESULTS IN ONE PARAGRAPH

Neurodevelopmental disorders with affected glutamate neurotransmission. Common elements underlying their pathophysiology can build the understanding of these pathologies, along with the description of new potential therapeutic targets.

Metabolism is an interesting prism, as it underlies in most pathologies and can provide a cross-sectional perspective to neurodevelopmental diseases.

We describe alterations regarding tryptophan metabolism which can be further studied for therapeutic purposes. Tryptophan and the importance of serotonin metabolism.

You describe SLC alterations which are the first new of such kind.

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

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). Studies have found that KYNA to be significantly increased in the cerebrospinal fluid (CSF) and brain tissue of patients with schizophrenia (Sathyasaikumar et al. 2011; Holtze et al. 2012)