**Figure 1. A) Glutamatergic neurotransmission alterations in disease cohorts.** Rett syndrome (MeCP2) and CDKL5-epileptic encephalopathy (CDKL5) were classified as hyperglutamatergic disorders , while GRIN-related pediatric encephalopathy (GRIN) and syntaxin encephalopathy (STXBP1) were classified as hypoglutamatergic disorders **B-F. Unsupervised multivariate analysis of patient and control CSF metabolite concentrations. PCA score plots showing the separation of patients from controls on the first two principal components.** B) Patients do not form distinct groups based on pathology or hyper/hypoglutamatergic alterations. Control samples are highly varied, although the majority separate from the patients.C) hyperglutamatergic patients. D) hypoglutamatergic patients. **Ward hierarchical clustering showing log-transformed metabolite concentrations.** Both E) hyperglutamatergic and F) hypoglutamatergic patients form homogeneous groups regardless of genotype. There is some overlap with controls, but mostly patients cluster together and have a markedly decreased concentration 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)** **A)** **hyperglutamatergic** and **B)** **hypoglutamatergic patients.** Both OPLS-DA models showed good separation between the groups (R2Y (cum) > 0.9, Q2Y (cum) > 0.8, RMSEE < 0.2), and both were statistically significant after permutation testing. **C-D) VIP scores.** Both diseases show similar metabolic alterations. **C) hyperglutamatergic** patients had 18 metabolites with a Variable Importance in Projection (VIP) score > 1 **D)** **hypoglutamatergic** patients had 17. The highest VIP scores in both cases belonged to tryptophan metabolites. **E-F) Integrated results of UVA and MVA analysis. E) hyperglutamatergic** patients **F) hypoglutamatergic patients.** For both groups there was a small number of metabolites identified as altered by both univariate and multivariate analyses. Both diseases showed similar metabolic alterations, though **hyperglutamatergic** (**C**) had a higher number of significantly altered metabolites. Interestingly, the **hyperglutamatergic** cohort showed significant alterations in metabolites that did not contribute to the performance of the OPLS-DA model. **G-H)** **Hierarchical clustering using only the selected metabolites** showed perfect separation between patients and controls for both groups. **G) Hyperglutamatergic patients** had increased concentrations of indole-3-propionic acid, galactose, and trigonelline, while **H) hypoglutamatergic patients** had increased concentrations of urea, trigonelline, and indole-3-propionic acid. but the rest of the altered metabolites all had decreased concentrations in patient cohorts.

**Figure 3. Pathways affected by selected metabolites.** Selected metabolites were converted to their KEGG compound IDs and mapped to their corresponding pathways. Only human pathways were taken into account, and pathways unrelated to the nervous system were filtered out. ABC transporters, galactose metabolism, tryptophan metabolism, and amino sugar and nucleotide sugar metabolism had the highest numbers of altered metabolites for both A) hyperglutamatergic, and B) hypoglutamatergic patients.

**Figure 4.** **Alterations in amino acid metabolism. A)** Summary of tryptophan metabolism pathways. **B) Alterations in tryptophan metabolism.** Of the metabolites involved in tryptophan metabolism that were analyzed, only 5-Hydroxyindole-3-acetic acid and kynurenine were significantly decreased in both disease groups, while kynurenic acid was significantly decreased in **hypoglutamatergic** patients and non-significantly decreased in hyperglutamatergic patients. Tryptophan and N-Acetyl-5-hydroxytryptamine showed a slight decrease that was not statistically significant in both hyperglutamatergic and hypoglutamatergic patients, while trigonelline was non-significantly increased in both groups. Interestingly, kynurenic acid was also significantly decreased in hypoglutamatergic patients compared to hyperglutamatergic patients **C) Alterations in other amino acids.** Phenylalanine was significantly decreased in both patient cohorts, while leucine was significantly decreased in hyperglutamatergic patients**.** Additionally, several amino acids (glycine, serine, isoleucine, and valine) were non-significantly decreased in both patient groups. It is noteworthy that glutamate was found to be significantly decreased only in hypoglutamatergic patients compared to hyperglutamatergic patients, but not between either patient group and controls.

**Figure 5.** **Tryptophan and BCAAs transport across the blood-brain barrier. A)** **Alterations in LNAAs in CSF samples**: Valine and leucine were decreased in patients compared to controls (statistically significant for hyperglutamatergic samples), while both groups had non-significantly altered levels of isoleucine and threonine. **B,C) Analysis of the expression of *SLC7A5* (LAT1) in brain samples from Rett mouse models.** Three different brain samples were analyzed for each group (Rett and controls) in two independent experiments. **B)** Representative blot of the expression of SLC7A5 is shown, where tubulin has been used as a loading control. **C)** Quantification with ImageJ of all the experiments; \*\* p-vale < 0.001

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| **ID** | **Disease group** | **Age at LP** | **Sex** | **5HIAA** (nmol/L) | **HVA** (nmol/L) | **HVA/5-HIAA** | **Gene** | **Mutation** | **Clinical presentation** | **Medications at time of lumbar puncture** |
| GRIN.1 | GRIN | 9 | F | **62 (87-366)** | 330 (202-596) | **5,32 (1,5-3,5)** | *GRIN2B*  NM\_015378 | p.(Pro553Thr) | Neurodevelopmental encephalopathy, global developmenta delay, profound intellectual disability, hypotonia, no ambulation, behaviour disorder. Partial epilepsy (clonic and myoclonic seizures). | Levopromazine, carbamazepine, melatonin, L-Serine |
| GRIN.2 | GRIN | 3 | M | **106 (106-316)** | **529 (304-658)** | 4,99 (1,5-3,5) | *GRIN2B* NM\_015378 | p.(Arg696His) | Neurodevelopmental encephalopathy, severe intellectual disability, autism, hyperkinetic behaviour, aggressiveness with autistic traits, multifocal epileptiform discharges without clinical seizures, hypotonia, walking with limitations | None |
| GRIN.3 | GRIN | 0 | F | **220 (217-1142)** | **439 (354-1328)** | 2 (1,5-3,5) | *GRIN1* NM\_007327 | p.(Gly641Arg) | Global developmenta delay, Severe intellectual disability, hyperkinesia, dyskinetic movements, hands-washing stereotypies, hypotonia, no ambulation. Partial epilepsy (oculocephalic crisis, onset at 5 months) that remitted with valproic acid | None |
| GRIN.5 | GRIN | 14 | M | **47 (63-185)** | **126 (156-410)** | 2,68 (1,5-3,5) | *GRIN2B* NM\_015378 | t (1;12) (1;12)(p13;q13) | Global developmenta delay, moderate intellectual disability, attention deficit hyperactivity disorder | Guanfacine, Melatonin |
| GRIN.6 | GRIN | 14 | F | **56 (63-185)** | **156 (156-410)** | 2,79 (1,5-3,5) | *GRIN2B* NM\_015378 | p.(Arg926\*) | Global developmenta delay, mild intellectual disability, multifocal epileptiform discharges without clinical seizures, attention deficit hyperactivity disorder | None |
| GRIN.7 | GRIN | 8 | M | 104 (87-366) | 377 (202 - 596) | **3,63 (1,5-3,5)** | *GRIN2B* NM\_015378 | p.(Pro553Thr) | Neurodevelopmental encephalopathy, global developmenta delay, profound intellectual disability, hypotonia, caregiver assistance walking, hyperkynetic movements, behaviour disorder Epileptic spasms | Levetiracetam |
| STXBP1.1 | STXBP1 | 5 | F | 124 (106-316) | 310 (304-658) | 2,5 (1,5-3,5) | *STXBP1* NM\_001032221 | p.(Arg406Cys) | Global developmental delay, congenital microcephaly. Epilepsy, generalized-onset and myoclonic seizure. Stereotypy. Limb dystonia and tremor. Severe intellectual disability. | Unknown |
| STXBP1.2 | STXBP1 | 3 | M | **164 (170-490)** | 578 (344-906) | **3,52 (1,5-3,5)** | *STXBP1* NM\_001032221 | p.(Met602Val) | Global developmental delay. Epilepsy, infantile spasms and generalized-onset seizures. Severe intellectual disability. | Levetiracetam, valproic acid, carbamazepine |
| STXBP1.3 | STXBP1 | 18 | F | 85 (63-185) | 289 (156-410) | 3,4 (1,5-3,5) | *STXBP1* NM\_001032221 | p.(Ile55Lys) | Infantile onset. Global developmental delay with prominent speech and language development. Autistic behavior. Intellectual disability, severe. Stereotypical body rocking . Behavioural abnormalities. | None |
| STXBP1.4 | STXBP1 | 9 | M | **70 (87-366)** | 344 (202-596 | **4,91 (1,5-3,5)** | *STXBP1* NM\_001032221 | p.(Gly417AlafsX7) | Neurodevelopmental delay. Generalized hypotonia. Postnatal microcephaly. Epileptic encephalopathy. Postnatal microcephaly. Action tremor. Nystagmus. Spastic tetraplegia Behavioral abnormality. | Valproic acid, clonazepam, pregabaline, rufinamide |
| CDKL5.1 | CDKL5 | 7 | F | 144 (106-316) | 434 (304-658) | 3,01 (1,5-3,5) | *CDKL5* NM\_001323289 | p.(Ser196Trp) | Early onset epileptic encephalopathy | Levetiracetam, vigabatrine |
| CDKL5.2 | CDKL5 | 8 | F | **65 (87-366)** | 267 (158-596) | **4,25 (1,5-3,5)** | *CDKL5* NM\_001323289 | p.(Gln902Stop) | Early onset epileptic encephalopathy (1st month). Neurodvelopmental delay detected at 5 months. Behavioural abnormalities with autolysis. | Carbamazepine |
| MECP2.1 | MECP2 | 9 | F | 176 (87-366) | 590 (158-596) | 3,35 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Glu137Gly) | Rett syndrome.Severe scoliosis and epliepsy | Valproic, levetiracetam |
| MECP2.2 | MECP2 | 3 | F | **117 (170-490)** | 493 (344-906) | **4,21 (1,5-3,5)** | *MECP2*  NM\_004992.4 | p.(Cys502Thr) | Rett syndrome. No eplipetic activity. Apnea and hyperpnea that are well controlled with topamax. | None |
| MECP2.3 | MECP2 | 9 | F | 123 (87-366) | 391 (158-596) | 3,18 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Arg255X) | Rett syndrome. Refractory epliepsy, dysphagia and severe apnea/yperpnea. Recurrent pneumonia. Died at 11 y.o. | Valproic acid, phenobarbital, clobazam |
| MECP2.4 | MECP2 | 6 | F | 242 (87-156) | 510 (184-464) | 2,11 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Lys321GlyfsX6) | Rett syndrome. Drug-responssive epilepsy. No scoliosis. Speech is preserved. | Zonisamide, Ethosuximide, risperidone |
| MECP2.5 | MECP2 | 4 | M | 162 (106-316) | 502 (304-658) | 3,10 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Arg309Trp) | Male with Rett syndrome (his mother was a carrier for the mutation). Epilepsy with debut at 3 years old. Ocular revulsion with generalized hypertonia and clonus of 4 limbs. | Valproic acid, risperidone, Levomepromazine |
| MECP2.6 | MECP2 | 6 | F | **90 (106-316)** | 292 (304-658) | 3,24 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Arg270X) | Rett syndrome. Spastic tetraparesia with possible mandibular dystonia. Pathologic EEG without clinical crisis. Left scoliosis. Repetitive pneumonias. Died at 17 y.o. |  |
| MECP2.7 | MECP2 | 5 | F | 210 (87-366) | 658 (237-596) | 3,13 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Arg306Cys) |  |  |
| MECP2.8 | MECP2 | 16 | F | 140 (82-140) | 336 (156-336) | 2,40 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Arg294X) |  | Carbamazepine |
| MECP2.9 | MECP2 | 11 | F | **65 (63-185)** | 298 (156-410) | **4,58 (1,5-3,5)** | *MECP2*  NM\_004992.4 | p.(Ser229Leu) | Rett syndrome. Daily crises of facial clonus of the right hemiface lasting up to 10 minutes. Scoliosis | Valproic acid, carbamazepine |
| MECP2.10 | MECP2 | 2 | F | **114 (170-490)** | 586 (344-906) | **5,14 (1,5-3,5)** | *MECP2*  NM\_004992.4 | p.(Arg255Stop) | Rett syndrome. Epilepsy. Scoliosis | Valproic acid, clobazam |
| MECP2.11 | MECP2 | 3 | F | **87 (170-490)** | **256 (344-906)** | 3,05 (1,5-3,5) | *MECP2*  NM\_004992.4 | p.(Thr158Met) | Rett syndrome. Pharmacorefractory epilepsy with polymorphic seizures, debut at the age of 4 years 11 months, uncontrolled. Mild global developmental delay. Hypovitaminosis D | None |
| MECP2.12 | MECP2 | 4 | F | **103 (106-316)** | 428 (304-658) | **4,16 (1,5-3,5)** | *MECP2*  NM\_004992.4 | p.(SerArg\_fsTer12) | Rett syndrome. Progressive microcephaly. Developmental regression. Sudden episodic apnea. Stereotypy. Autistic behavior. Global developmental delay  Strabismus. EEG abnormality | Topiramate |

**Table 1: Patients major findings and description summary.** Description of patient characteristics at the time of lumbar puncture**.** Age at LP: age at the time lumbar puncture was taken; Sex: M=Male, F=Female; 5HIAA= 5-Hydroxyindoleacetic acid, HVA=Homovanillic acid (these were measured at the time of lumbar puncture by HPLC); Gene = common name and RefSeq accession number of mutated gene.