Utilization of the Biolog Platform to Understand Neurodevelopmental Disorders

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Where Compassion Inspires Progress

BIOLOG PLATES

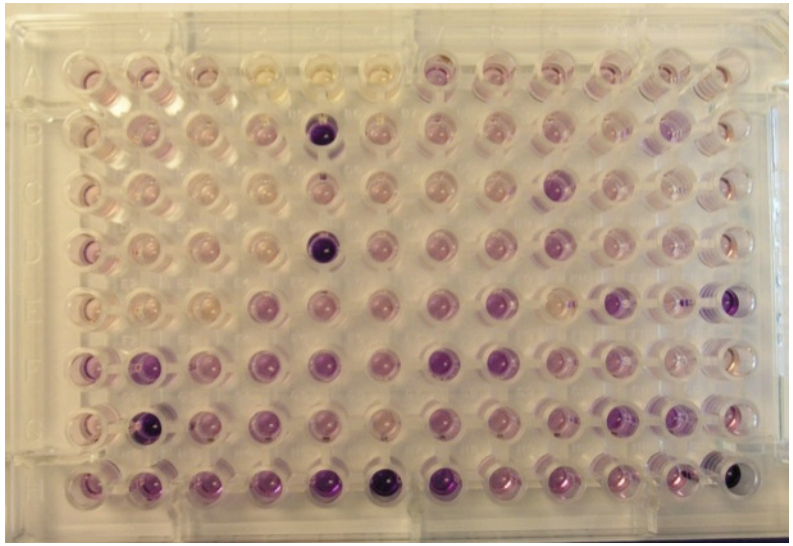
- 96-well plates
- 50 µl/well of cell suspension (20,000 cells/well)
- Incubate 40-48 hours, add Redox Dye Mix (tetrazolium) and incubate for 24 hours
- Plate substrates:

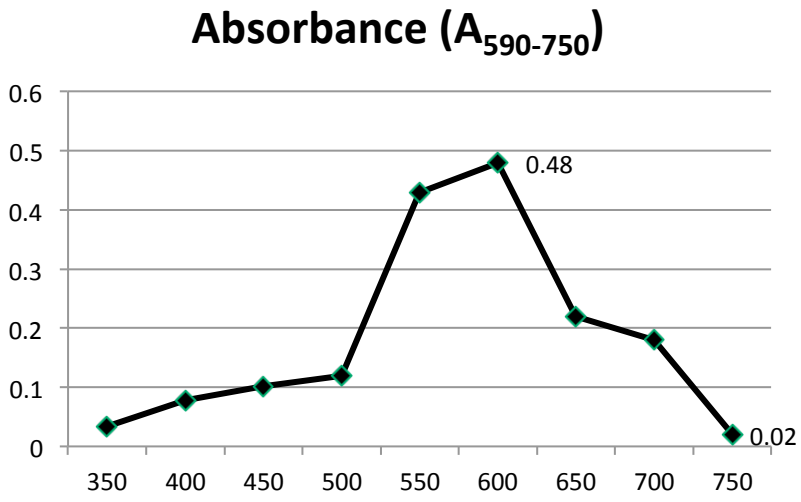
    1. Carbon energy sources (sugars, polysaccharides, nucleotides, and carboxylic acids)

    2-4. Amino acids and dipeptides

    5. Ions

    6-8. Hormones and metabolic effectors





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BIOLOG studies

(in bold significant results)

ASDs (151 cases)

- ***NLGN4*** **(2)**
- ***SHANK3*** **(3)**
- ***FMR1*** **(4)**
- ***MECP2*** **(2)**
- ***ZNF711*** **(1)**
- **Other (128)**

Schizophrenia (10 cases)

XLID (60 cases)

- ***MED12*** **(3 FG and 1 Lujan)**
- ***UPF3B*** **(3)**
- ***SMS*** **(8)**
- ***FMR1*** **(8+4, no ASDs)**
- ***ACSL4*** **(1)**
- ***SLC6A8*** **(1)**
- ***SLC9A6*** **(1)**
- ***MECP2-L1CAM dup*** **(2)**
- ***ZNF711*** **(1)**
- *ATRX* (1)
- *RSK2* (1)
- *FGD1* (14)
- *L1CAM* (1)
- *MCT8* (1)

Autosomal ID (5 cases)

- ***ZBTB20*** **(2)**
- ***ST3GAL5*** **(1)**
- Angelman (2)

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Autism Spectrum Disorders (ASDs)

- ASDs include a series of conditions characterized by delays or abnormal functioning before the age of 3 in one or more of the following domains: **social interaction**; **communication**; restricted, repetitive, and stereotyped **patterns of behavior, interests, and activities**.
- The main disorders of the spectrum (DSM-IV-TR) are:
  - ✓ **Autistic disorder**
  - ✓ **Asperger syndrome**
  - ✓ **Pervasive developmental disorder not otherwise specified (PDD-NOS)**.
- Prevalence: 1/88 (US Center for Disease Control and Prevention, 2012).
- ASD research funding in US (2010): $408.5 M, (+84% from 2008).

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Significant metabolites in 10 non-syndromic ASD patients *vs.* 10 controls

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	
Substrate	15627 (*NLGN4* A289T)	16309 (*SHANK3* c. 1304+48 C>T + microdel 18)	17702 (*NLGN4* S259P)	CMS 13961 (twin)	CMS 13963 (twin)	CMS 17711	CMS 4613	12718 (*SHANK3* c. 1304+48 C>T + *EGR2* SNPs)	16453 (*SHANK3* c. 1304+48 C>T)	CMS 6693 (t 14;15)	***P* value**
Trp-Arg	0.123	0.223	0.183	0.188	0.236	0.296	0.249	0.276	0.238	0.277	0.00014
Trp-Glu	0.133	0.277	0.203	0.225	0.271	0.323	0.317	0.437	0.251	0.29	0.00028
L-Tryptophan	0.215	0.378	0.305	0.303	0.377	0.293	0.44	0.344	0.394	0.447	0.00028
Trp-Gly	0.139	0.28	0.212	0.222	0.236	0.258	0.266	0.389	0.363	0.337	0.00042
Trp-Trp	0.195	0.297	0.302	0.25	0.337	0.296	0.35	0.433	0.408	0.312	0.00049
Ala-Trp	0.179	0.269	0.219	0.24	0.311	0.326	0.325	0.319	0.288	0.333	0.00066
Asp-Trp	0.138	0.256	0.199	0.219	0.25	0.29	0.268	0.27	0.221	0.402	0.00071
Phe-Trp	0.19	0.229	0.262	0.239	0.297	0.261	0.274	0.4	0.338	0.32	0.00189
Met-Trp	0.18	0.302	0.323	0.335	0.417	0.302	0.371	0.325	0.383	0.436	0.00210
Glu-Trp	0.148	0.286	0.247	0.296	0.348	0.301	0.359	0.32	0.255	0.55	0.00305
Trp-Asp	0.124	0.257	0.171	0.197	0.273	0.346	0.245	0.396	0.259	0.28	0.00353
Leu-Trp	0.171	0.326	0.205	0.301	0.418	0.268	0.322	0.339	0.472	0.359	0.00459
Trp-Ala	0.158	0.273	0.222	0.235	0.33	0.314	0.345	0.438	0.347	0.416	0.00466
Trp-Tyr	0.132	0.303	0.211	0.263	0.336	0.254	0.309	0.408	0.342	0.318	0.00673
Trp-Val	0.159	0.309	0.185	0.274	0.338	0.248	0.336	0.412	0.286	0.348	0.00804
Ile-Trp	0.166	0.311	0.246	0.293	0.348	0.316	0.373	0.363	0.355	0.473	0.00961
Arg-Trp	0.127	0.181	0.14	0.169	0.218	0.342	0.205	0.249	0.345	0.27	0.01054
Trp-Leu	0.195	0.331	0.227	0.284	0.384	0.242	0.297	0.361	0.464	0.335	0.01984
Lys-Lys	0.096	0.093	0.076	0.102	0.115	0.243	0.102	0.207	0.192	0.273	0.02204
Leu-Ile	0.167	0.116	0.081	0.207	0.266	0.408	0.234	0.306	0.371	0.403	0.02210
Trp-Phe	0.181	0.355	0.293	0.308	0.418	0.253	0.3	0.414	0.369	0.293	0.02273
Ile-Tyr	0.099	0.164	0.1	0.151	0.185	0.356	0.187	0.268	0.19	0.309	0.02423
His-Trp	0.128	0.241	0.178	0.226	0.247	0.304	0.229	0.222	0.255	0.318	0.02525
Gly-Trp	0.138	0.298	0.253	0.316	0.349	0.363	0.357	0.276	0.333	0.309	0.02582
Trp-Ser	0.166	0.317	0.232	0.255	0.298	0.226	0.323	0.372	0.398	0.311	0.02632
a-Keto-Butyric Acid	0.113	0.114	0.111	0.159	0.094	0.106	0.157	0.189	0.226	0.422	0.02740
Mono Methyl Succinate	1.236	1.54	2.116	1.286	1.664	0.714	1.048	1.95	1.545	1.412	0.03126
Ile-Gln	0.385	0.8	0.544	0.625	0.977	0.426	0.7	0.849	0.792	0.835	0.03223
L-Lactic Acid (DL)	0.464	0.572	0.867	0.666	0.922	0.706	0.318	0.588	0.823	0.797	0.03291
D,L-a-Glycerol Phosphate	0.408	0.328	0.516	0.252	0.236	0.409	0.247	0.624	0.37	0.496	0.03566
His-Glu	0.082	0.093	0.072	0.096	0.105	0.233	0.116	0.156	0.177	0.2	0.03605
Tyr-Gly	0.102	0.138	0.103	0.134	0.155	0.235	0.147	0.308	0.269	0.261	0.03918
His-Val	0.08	0.083	0.072	0.101	0.09	0.28	0.086	0.199	0.126	0.26	0.04165
a-Methyl-D-Mannoside	0.118	0.172	0.149	0.216	0.401	0.524	0.221	0.719	0.426	0.249	0.04571

Substrate	Control Average	Green Average	Orange Average	Purple Average	Z Average	*P*-Value
Glycogen	1.489	1.164	1.161	0.911	1.402	0.026949
L-Lactic Acid (DL)	0.479	0.695	0.769	0.556	0.657	0.006211
Succinamic Acid	1.626	1.298	1.422	0.812	1.651	0.023753
Mono Methyl Succinate	1.884	1.475	1.671	0.992	1.819	0.011704
b-Hydroxy-Butyric Acid	0.359	0.529	0.557	0.379	0.412	0.023910
Hexanoic Acid	0.338	0.489	0.554	0.415	0.390	0.011992
L-Tryptophan	0.631	0.365	0.375	0.241	0.377	0.001461
Ala-Arg	0.194	0.273	0.314	0.222	0.211	0.018475
Ala-Trp	0.472	0.292	0.298	0.203	0.299	0.001675
Arg-Trp	0.357	0.235	0.238	0.165	0.232	0.001373
Asp-Trp	0.449	0.264	0.314	0.166	0.242	0.001115
Glu-Trp	0.532	0.329	0.342	0.178	0.330	0.000942
Gly-Trp	0.462	0.317	0.297	0.184	0.299	0.006416
Gly-Tyr	0.277	0.201	0.231	0.146	0.189	0.015515
His-Trp	0.379	0.247	0.254	0.187	0.249	0.004921
His-Tyr	0.238	0.171	0.241	0.115	0.161	0.031716
Ile-Gln	1.021	0.728	0.979	0.904	0.611	0.029556
Ile-Trp	0.538	0.342	0.397	0.278	0.324	0.006989
Ile-Tyr	0.331	0.212	0.313	0.216	0.209	0.018489
Leu-Phe	0.190	0.241	0.371	0.338	0.189	0.039625
Leu-Trp	0.498	0.334	0.371	0.372	0.320	0.002536
Met-Trp	0.528	0.355	0.359	0.248	0.348	0.001670
Met-Tyr	0.310	0.225	0.275	0.168	0.215	0.021180
Phe-Trp	0.395	0.291	0.337	0.203	0.288	0.000191
Pro-Trp	0.500	0.406	0.367	0.311	0.337	0.021670
Ser-Tyr	0.301	0.214	0.266	0.185	0.212	0.013745
Trp-Ala	0.486	0.324	0.321	0.223	0.309	0.001209
Trp-Arg	0.402	0.241	0.290	0.158	0.231	0.000173
Trp-Asp	0.412	0.269	0.318	0.141	0.225	0.000740
Trp-Glu	0.494	0.288	0.352	0.162	0.255	0.000132
Trp-Gly	0.472	0.285	0.317	0.190	0.268	0.000299
Trp-Leu	0.447	0.325	0.309	0.212	0.314	0.001800
Trp-Lys	0.399	0.310	0.276	0.163	0.279	0.014358
Trp-Phe	0.420	0.334	0.312	0.231	0.295	0.000590
Trp-Ser	0.501	0.304	0.297	0.236	0.291	0.004266
Trp-Trp	0.505	0.332	0.320	0.219	0.341	0.000153
Trp-Tyr	0.472	0.305	0.295	0.179	0.287	0.001164
Trp-Val	0.481	0.304	0.344	0.195	0.310	0.003150
Tyr-Ala	0.314	0.210	0.270	0.157	0.200	0.036694
Tyr-Gly	0.287	0.194	0.244	0.088	0.176	0.005801
Tyr-Ile	0.347	0.230	0.295	0.155	0.200	0.019385
Tyr-Phe	0.252	0.200	0.239	0.131	0.145	0.027475
Tyr-Trp	0.336	0.253	0.278	0.157	0.231	0.002382
Tyr-Tyr	0.328	0.233	0.225	0.124	0.185	0.003382
Tyr-Val	0.293	0.210	0.242	0.116	0.154	0.014950
Val-Gln	0.731	0.499	0.615	0.543	0.380	0.022287
Val-Tyr	0.303	0.229	0.273	0.138	0.187	0.024195

25/26 (96.1%) Trp ($p<0.05$)
(only Lys-Trp missing, but it is in acetate salt)

15627 (*NLGN4* A289T)	G1
16309 (*SHANK3* c.1304+48C>T + microdel 18)	G2
17702 (*NLGN4* S259P)	G3
cms13961 (twin)	G4
cms13963 (twin)	G5
cms17711	G6
cms4613	G7
12718 (*SHANK3* c.1304+48C>T + *EGR2* SNPs)	G8
16453 (*SHANK3* c.1304+48C>T)	G9
CMS 6693 (t 14;15)	G10
CMS 6276a *FMR1*	O1
CMS 9549 *FMR1*	O2
CMS 15810 *FMR1*	O3
CMS 15811 *FMR1*	O4
CMS 1836 (*MECP2* R168X)	P1
CMS 3294 (*MECP2* M158T)	P2
14071 (*ZNF711*)	Z1

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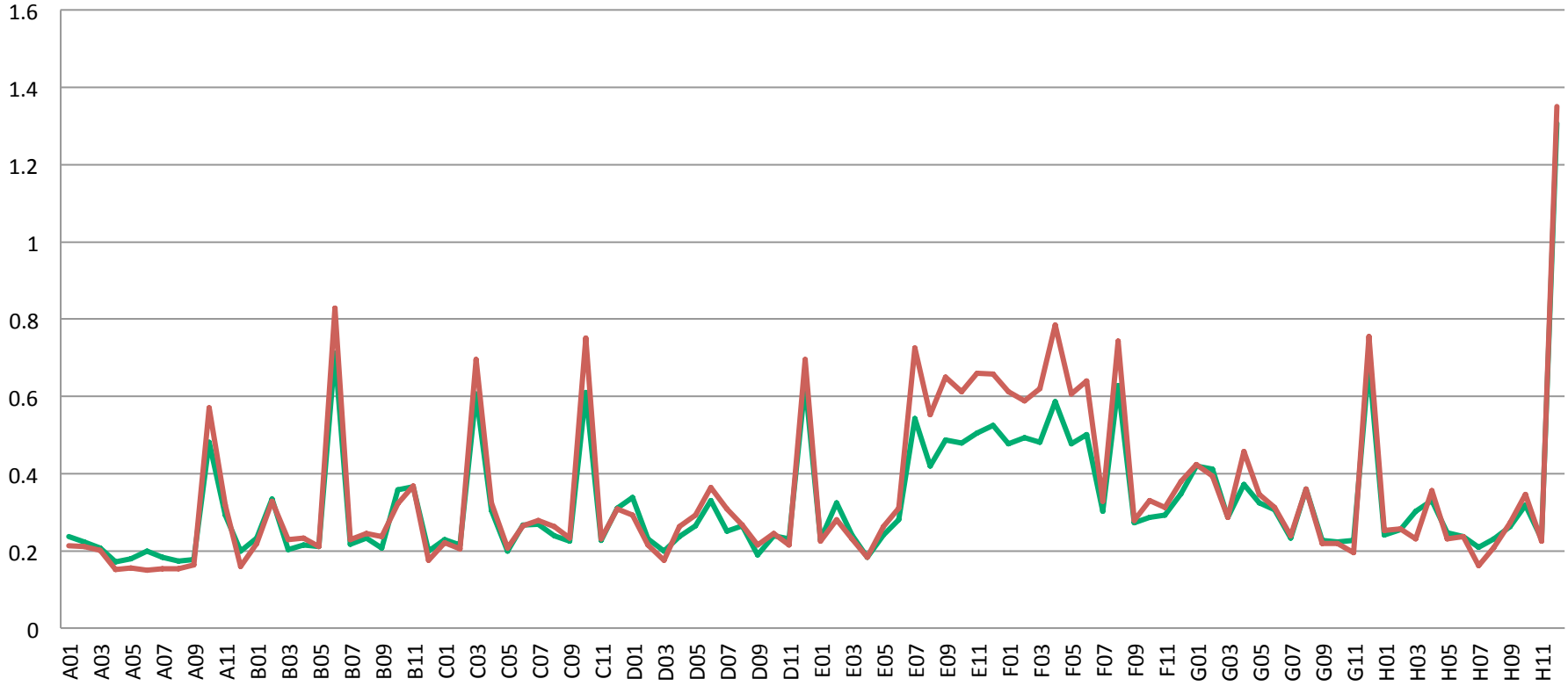
Average of 20 ASD patients *vs.* 20 controls



Trp [E7-F6] Av 20 ctrs	Trp [E7-F6] Av 20 pts	Diff: -22.55%
0.6428375	0.497841667	

Preliminary microarray data

- ✓ Expression microarray analysis on 10 ASD patients vs 10 controls showed significantly lower expression of 2 out of 3 genes responsible for the Trp receptors expressed both in blood and brain (*SLC7A5* and *SLC7A8*), the data was confirmed by qPCR

Gene Symbol	*P* Value
SLC3A2	0.25619
SLC7A5	0.00627
SLC7A8	0.04067

- ✓ The same microarray analysis also showed significant abnormalities in the expression of several enzymes of the Trp-Kynurenine pathway

Gene Symbol	*P* value
AADAT	0.0022
HAAO	0.0158
MAOA	0.0027
TPH2	0.0166

Data trends for singular Autism patients

AADAT= DOWN 7/10	*QPRT*= UP 7/10
MAOA= DOWN 7/10	
TDO2= DOWN 5/10 + UP 1/10	
HAAO= DOWN 4/10	
TPH2= DOWN 4/10	

- ✓ The mitochondrial form of the gene responsible for Trp tRNA was under-expressed, while the cytoplasmic form did not significant differences

Gene Symbol	*P* value
WARS (cytoplasmic form)	0.35496095
WARS2 (mitochondrial form)	0.01599819

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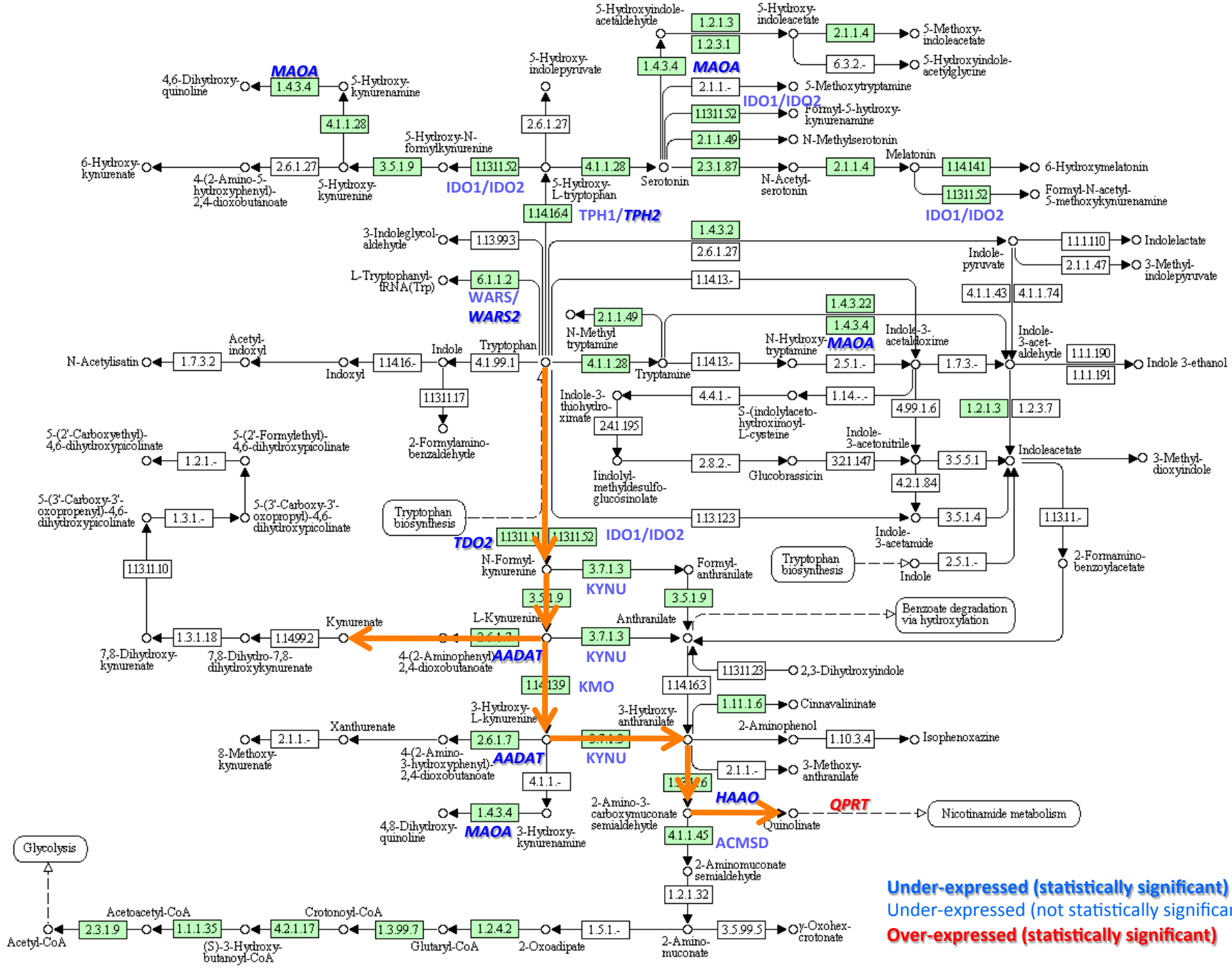
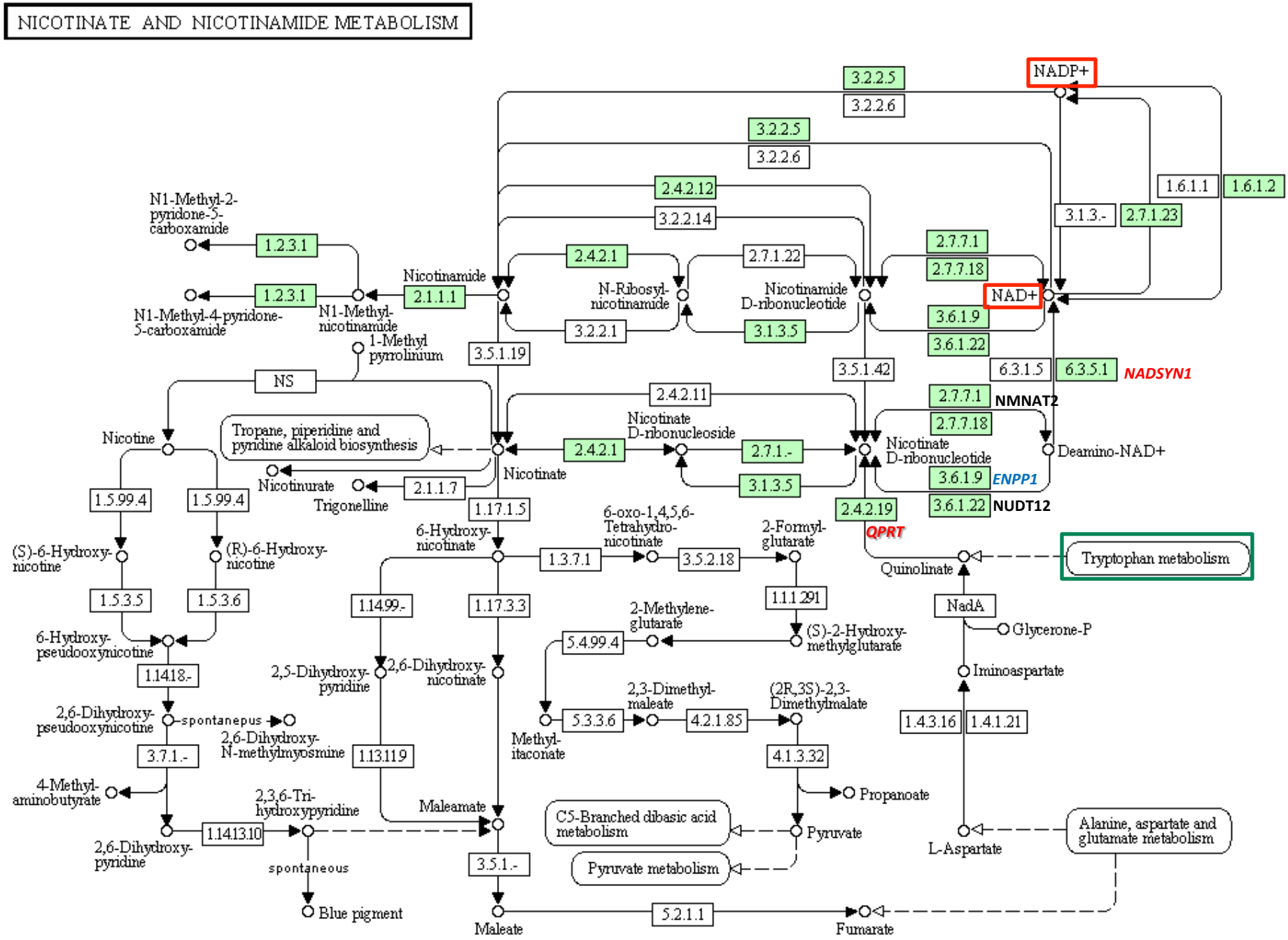

TRYPTOPHAN METABOLISM
MAOA
IDO1/IDO2
TPH1/TPH2
WARS/
WARS2
TDO2
KYNU
AADAT
KMO
HAAO
QPRT
ACMSD
Tryptophan biosynthesis
Benzoate degradation via hydroxylation
Nicotinamide metabolism
Glycolysis
Under-expressed (statistically significant)
Under-expressed (not statistically significant)
Over-expressed (statistically significant)


NICOTINATE AND NICOTINAMIDE METABOLISM
NADP+
NAD+
NADSYN1
NMNAT2
ENPP1
NUDT12
QPRT
Nicotinamide
N1-Methyl-2-pyridone-5-carboxamide
N1-Methyl-4-pyridone-5-carboxamide
N1-Methyl-nicotinamide
1-Methyl pyrrolinium
NS
N-Ribosyl-nicotinamide
Nicotinamide D-ribonucleotide
Nicotinamide D-ribonucleoside
Nicotinate D-ribonucleotide
Deamino-NAD+
Nicotine
Tropane, piperidine and pyridine alkaloid biosynthesis
Nicotinate
Nicotinurate
Trigonelline
(S)-6-Hydroxy-nicotine
(R)-6-Hydroxy-nicotine
6-Hydroxy-nicotinate
6-oxo-1,4,5,6-Tetrahydro-nicotinate
2-Formyl-glutarate
Quinolinate
Tryptophan metabolism
NadA
Glycerone-P
Iminoaspartate
L-Aspartate
Alanine, aspartate and glutamate metabolism
6-Hydroxy-pseudooxynicotine
2,6-Dihydroxy-pseudooxynicotine
spontanepus
2,6-Dihydroxy-N-methylmyosmine
2,5-Dihydroxy-pyridine
2,6-Dihydroxy-nicotinate
2-Methylene-glutarate
(S)-2-Hydroxy-methylglutarate
2,3-Dimethyl-maleate
(2R,3S)-2,3-Dimethylmalate
Methyl-itaconate
4-Methyl-aminobutyrate
2,3,6-Tri-hydroxypyridine
2,6-Dihydroxy-pyridine
spontaneous
Blue pigment
Maleamate
Maleate
Fumarate
Propanoate
Pyruvate
C5-Branched dibasic acid metabolism
Pyruvate metabolism
3.2.2.5
3.2.2.6
2.4.2.12
3.2.2.14
1.6.1.1
1.6.1.2
3.1.3.-
2.7.1.23
1.2.3.1
2.4.2.1
2.7.1.22
2.7.7.1
2.7.7.18
2.1.1.1
3.2.2.1
3.1.3.5
3.6.1.9
3.6.1.22
3.5.1.19
3.5.1.42
6.3.1.5
6.3.5.1
2.4.2.11
2.7.1.-
2.1.1.7
1.5.99.4
1.5.3.5
1.5.3.6
1.17.1.5
1.3.7.1
3.5.2.18
2.4.2.19
1.1.1.291
1.14.99.-
1.17.3.3
5.4.99.4
1.14.18.-
5.3.3.6
4.2.1.85
4.1.3.32
1.4.3.16
1.4.1.21
3.7.1.-
1.13.11.9
1.14.13.10
3.5.1.-
5.2.1.1

Background and Hypothesis

Trp->Kynurenine: the balance between kynurenic acid and quinolinic acid (final products of the kynurenine pathway) is critical for **neuronal growth, maturation** and **apoptosis**

Quinolinic acid: neurotoxic, induces **mitochondrial dysfunction** and increases free radical generation, is a **potent NMDA agonist**, maybe the endogenous ligand during early programmed **apoptosis**

Kynurenic acid: neuroprotective, **NMDA antagonist**, and **prevents glutamate-induced neuronal death**, particularly in the hippocampus

Nitric oxide (NO): influences expression of both molecules, plays a critical role in the interaction between **inflammation and neuronal circuits**, causes **mitochondrial dysfunction**, and has been reported elevated in ASD patients

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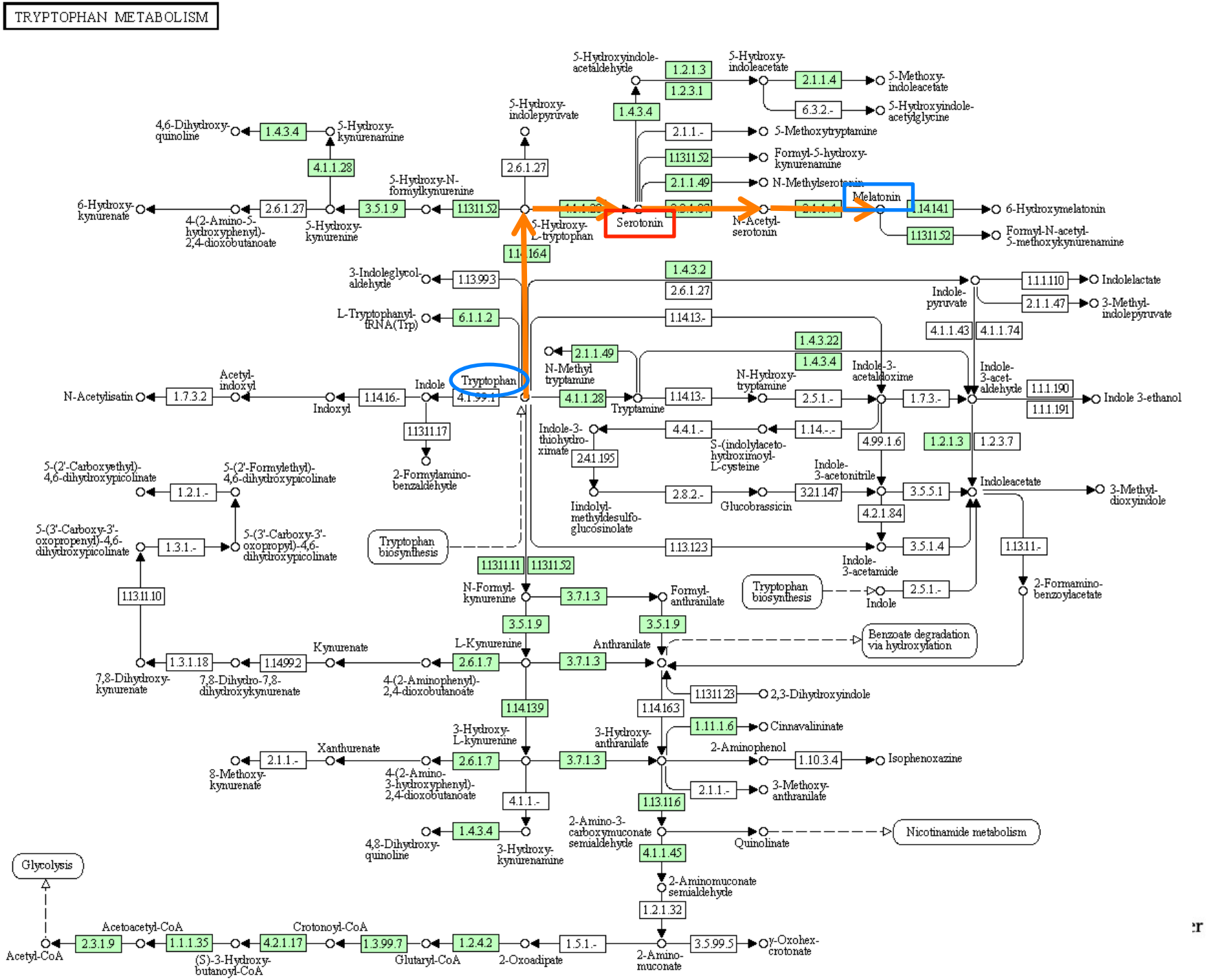
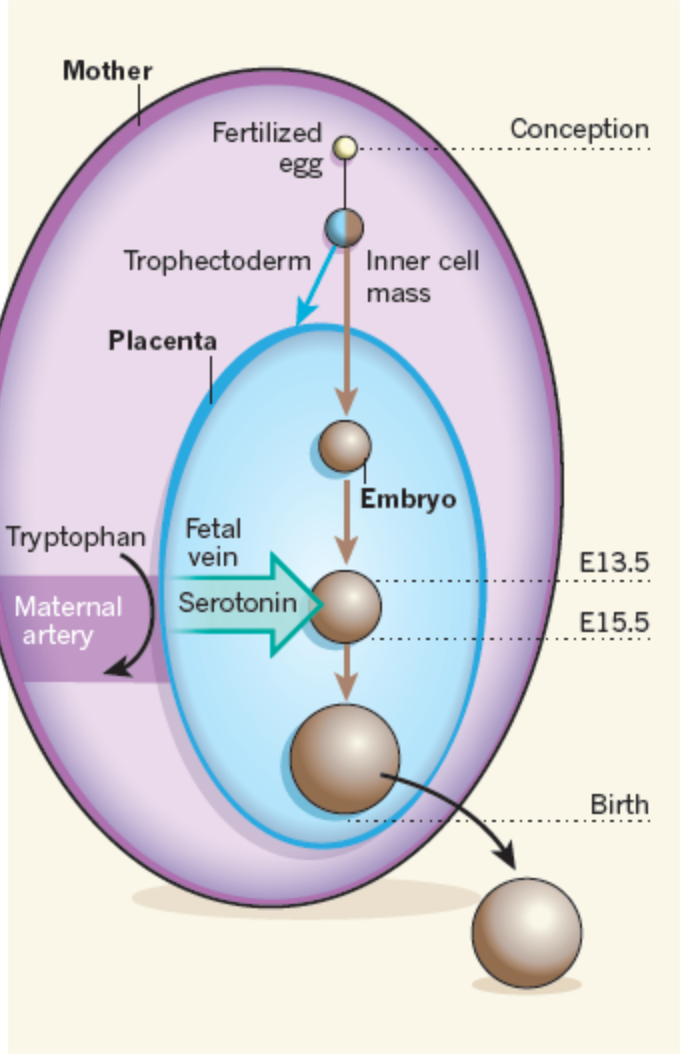

TRYPTOPHAN METABOLISM
Tryptophan
Serotonin
Melatonin
5-Hydroxy-L-tryptophan
5-Hydroxyindolepyruvate
5-Hydroxyindoleacetaldehyde
5-Hydroxyindoleacetate
5-Methoxyindoleacetate
5-Hydroxyindoleacetylglycine
5-Methoxytryptamine
Formyl-5-hydroxykynurenamine
N-Methylserotonin
N-Acetylserotonin
6-Hydroxymelatonin
Formyl-N-acetyl-5-methoxykynurenamine
5-Hydroxy-N-formylkynurenine
5-Hydroxykynurenine
5-Hydroxykynurenamine
4,6-Dihydroxyquinoline
6-Hydroxykynurenate
4-(2-Amino-5-hydroxyphenyl)-2,4-dioxobutanoate
3-Indoleglycolaldehyde
L-Tryptophanyl-tRNA(Trp)
Indolepyruvate
Indolelactate
3-Methylindolepyruvate
N-Methyltryptamine
N-Hydroxytryptamine
Indole-3-acetaldoxime
Indole-3-acetaldehyde
Indole 3-ethanol
Tryptamine
Indole
Indoxyl
Acetylindoxyl
N-Acetylisatin
2-Formylaminobenzaldehyde
Indole-3-thiohydroximate
S-(indolylacetohydroximoyl)-L-cysteine
Indole-3-acetonitrile
Indolylmethyldesulfoglucosinolate
Glucobrassicin
Indoleacetate
3-Methyldioxyindole
Indole-3-acetamide
5-(2'-Carboxyethyl)-4,6-dihydroxypicolinate
5-(2'-Formylethyl)-4,6-dihydroxypicolinate
5-(3'-Carboxy-3'-oxopropenyl)-4,6-dihydroxypicolinate
5-(3'-Carboxy-3'-oxopropyl)-4,6-dihydroxypicolinate
Tryptophan biosynthesis
N-Formylkynurenine
Formylanthranilate
Kynurenate
L-Kynurenine
Anthranilate
Benzoate degradation via hydroxylation
7,8-Dihydroxykynurenate
7,8-Dihydro-7,8-dihydroxykynurenate
4-(2-Aminophenyl)-2,4-dioxobutanoate
2,3-Dihydroxyindole
3-Hydroxy-L-kynurenine
3-Hydroxyanthranilate
Cinnavalininate
2-Aminophenol
Isophenoxazine
Xanthurenate
8-Methoxykynurenate
4-(2-Amino-3-hydroxyphenyl)-2,4-dioxobutanoate
3-Methoxyanthranilate
4,8-Dihydroxyquinoline
3-Hydroxykynurenamine
2-Amino-3-carboxymuconate semialdehyde
Quinolinate
Nicotinamide metabolism
2-Aminomuconate semialdehyde
2-Aminomuconate
γ-Oxohexcrotonate
Glycolysis
Acetoacetyl-CoA
Acetyl-CoA
(S)-3-Hydroxybutanoyl-CoA
Crotonoyl-CoA
Glutaryl-CoA
2-Oxoadipate
2-Formamidobenzoylacetate

Background and Hypothesis

Trp->Serotonin: Trp depletion can cause **abnormal behavior**, Serotonin (5-HT) has been associated with **mood and behavioral regulation**. During the first trimester of gestation, 5-HT is produced by the placenta starting from maternal Trp. This "endogenous" 5-HT is critical in the **development of frontal lobes** and in the organization of **glutamatergic synapses**.



from McKay, Nature 2011

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Frequency of metabolites that are selected by 7 different feature selection algorithms after testing 17 ASD patients *vs.* 18 controls

ChiSquared	InfoGain	GainRatioAttribute	SymmetricalUncert	OneRAttribute	RelieFAttribute	SVM
Trp-Ala	**Trp-Ala**	**Trp-Ala**	**Trp-Ala**	Trp-Ala	Trp-Asp	Trp-Ala
Trp-Arg	**Trp-Arg**	**Trp-Arg**	**Trp-Arg**	Trp-Asp	Trp-Gly	Trp-Arg
Trp-Glu	**Trp-Glu**	**Trp-Glu**	**Trp-Glu**	Trp-Gly	Trp-Ser	Trp-Asp
Trp-Gly	**Trp-Gly**	**Trp-Gly**	**Trp-Gly**	Trp-Lys	Trp-Tyr	Trp-Ser
Trp-Leu	**Trp-Leu**	**Trp-Leu**	**Trp-Leu**	Trp-Tyr	Trp-Val	Trp-Trp

Frequency of metabolites that are selected by the 10 different feature selection algorithms after testing 37 ASD patients *vs.* 20 controls

Count	Metabolites	Frequency	Comment
1	Trp-Gly	10	Recommended
2	Trp-Lys	8	Recommended; new on the list
3	Trp-Ala	7	Recommended
4	Trp-Arg	7	Recommended
5	Trp-Leu	5	Recommended
6	Trp-Phe	4	Considered; new on the list

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Customized Biolog Tryptophan plate

A1 α-D-Glucose	A2 α-D-Glucose	A3 α-D-Glucose	A4 α-D-Glucose	A5 α-D-Glucose	A6 α-D-Glucose	A7 α-D-Glucose	A8 α-D-Glucose	A9 α-D-Glucose	A10 α-D-Glucose	A11 α-D-Glucose	A12 α-D-Glucose
B1 Empty	B2 Empty	B3 Empty	B4 Empty	B5 Empty	B6 Empty	B7 Empty	B8 Empty	B9 Empty	B10 Empty	B11 Empty	B12 Empty
C1 L-Tryptophan	C2 L-Tryptophan	C3 L-Tryptophan	C4 L-Tryptophan	C5 L-Tryptophan	C6 L-Tryptophan	C7 L-Tryptophan	C8 L-Tryptophan	C9 L-Tryptophan	C10 L-Tryptophan	C11 L-Tryptophan	C12 L-Tryptophan
D1 Trp-Gly	D2 Trp-Gly	D3 Trp-Gly	D4 Trp-Gly	D5 Trp-Gly	D6 Trp-Gly	D7 Trp-Gly	D8 Trp-Gly	D9 Trp-Gly	D10 Trp-Gly	D11 Trp-Gly	D12 Trp-Gly
E1 Trp-Lys	E2 Trp-Lys	E3 Trp-Lys	E4 Trp-Lys	E5 Trp-Lys	E6 Trp-Lys	E7 Trp-Lys	E8 Trp-Lys	E9 Trp-Lys	E10 Trp-Lys	E11 Trp-Lys	E12 Trp-Lys
F1 Trp-Ala	F2 Trp-Ala	F3 Trp-Ala	F4 Trp-Ala	F5 Trp-Ala	F6 Trp-Ala	F7 Trp-Ala	F8 Trp-Ala	F9 Trp-Ala	F10 Trp-Ala	F11 Trp-Ala	F12 Trp-Ala
G1 Trp-Arg	G2 Trp-Arg	G3 Trp-Arg	G4 Trp-Arg	G5 Trp-Arg	G6 Trp-Arg	G7 Trp-Arg	G8 Trp-Arg	G9 Trp-Arg	G10 Trp-Arg	G11 Trp-Arg	G12 Trp-Arg
H1 Trp-Leu	H2 Trp-Leu	H3 Trp-Leu	H4 Trp-Leu	H5 Trp-Leu	H6 Trp-Leu	H7 Trp-Leu	H8 Trp-Leu	H9 Trp-Leu	H10 Trp-Leu	H11 Trp-Leu	H12 Trp-Leu

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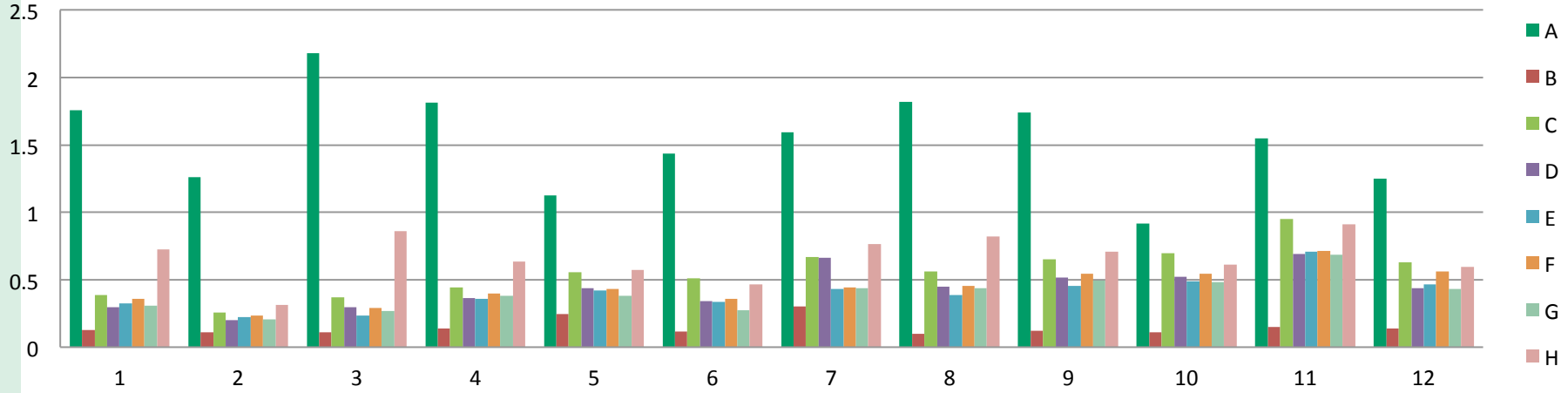
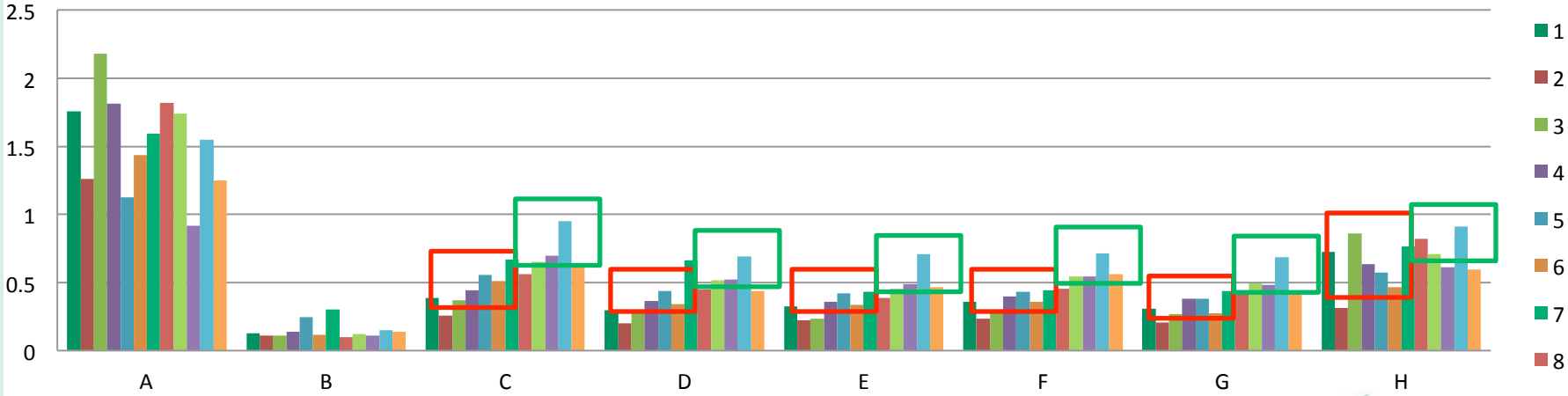
New plate 6 ASDs *vs.* 6 Controls



New plate 6 ASDs *vs.* 6 Controls



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New customized Biolog tryptophan plate
6 ASDs *vs.* 6 Controls

Substrate	Aut 15057	Aut 13669	Aut 18454	Aut 15301	Aut 17160	Aut CMS 14869	Control CMS 11237	Control CMS 11959	Control CMS 11210	Control CMS 12225	Control CMS 12223	Control CMS 12808	*P* value (*t* test)
a-D-Glucose	1.755	1.259	2.182	1.815	1.124	1.436	1.596	1.817	1.743	0.916	1.549	1.247	0.6205
Empty	0.129	0.109	0.108	0.141	0.247	0.116	0.303	0.098	0.12	0.111	0.147	0.138	0.8271
L-Trptophan	**0.384**	**0.259**	**0.37**	**0.443**	**0.555**	**0.513**	**0.67**	**0.564**	**0.651**	**0.696**	**0.949**	**0.627**	**0.0041**
Trp-Gly	**0.297**	**0.2**	**0.296**	**0.366**	**0.435**	**0.339**	**0.664**	**0.448**	**0.519**	**0.524**	**0.694**	**0.437**	**0.0028**
Trp-Lys	**0.323**	**0.221**	**0.237**	**0.356**	**0.421**	**0.334**	**0.433**	**0.389**	**0.454**	**0.486**	**0.706**	**0.466**	**0.0068**
Trp-Ala	**0.359**	**0.234**	**0.289**	**0.398**	**0.434**	**0.361**	**0.441**	**0.457**	**0.543**	**0.542**	**0.716**	**0.56**	**0.0032**
Trp-Arg	**0.308**	**0.204**	**0.269**	**0.383**	**0.38**	**0.274**	**0.439**	**0.436**	**0.494**	**0.48**	**0.683**	**0.43**	**0.0024**
Trp-Leu	0.727	0.315	0.863	0.635	0.571	0.468	0.763	0.821	0.708	0.611	0.913	0.595	0.1687

Note: the *t* test was performed on the log-transformed data.

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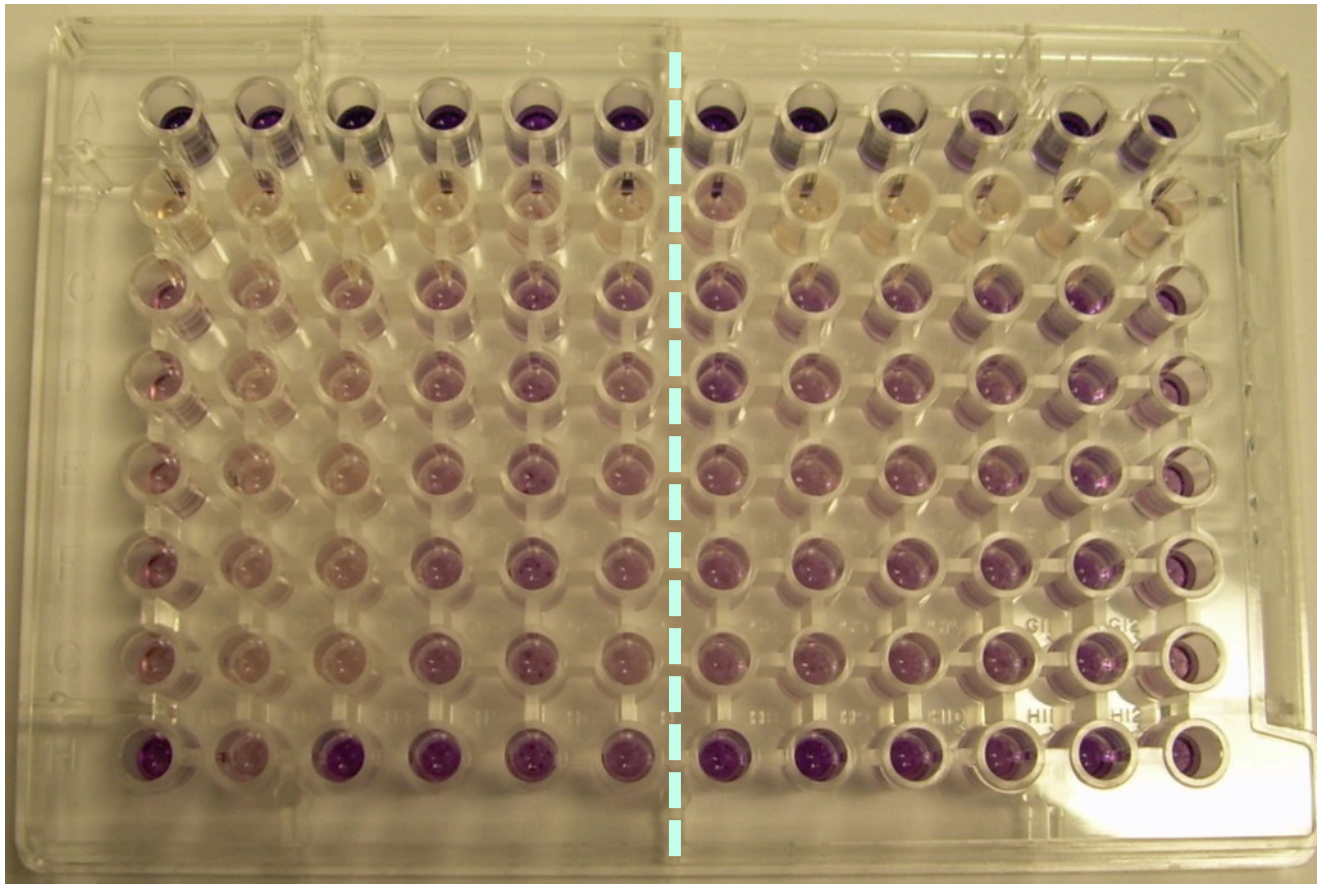
New customized Biolog tryptophan plate
6 ASDs *vs.* 6 Controls



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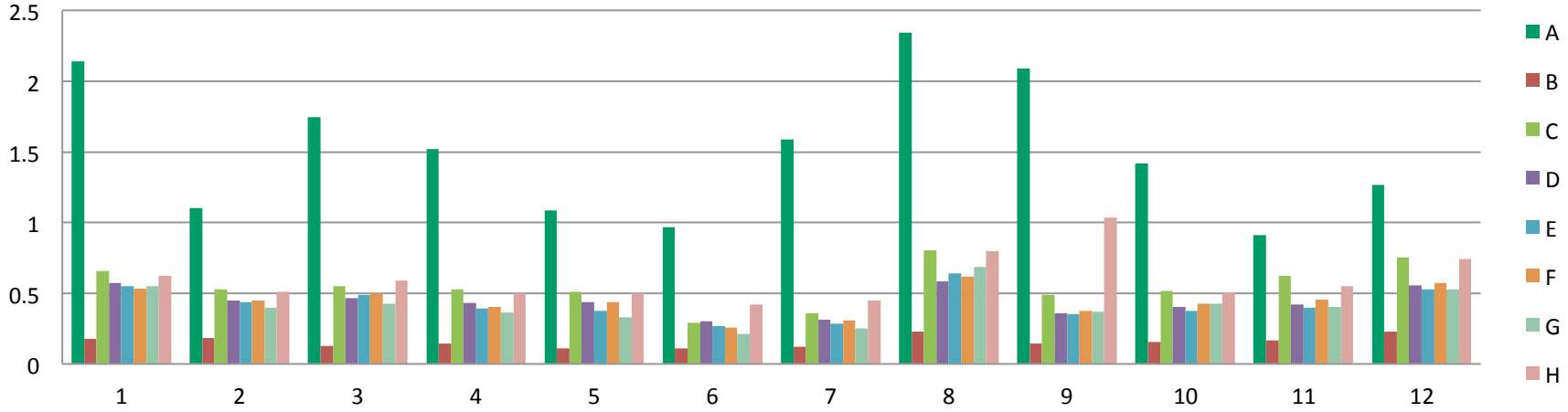
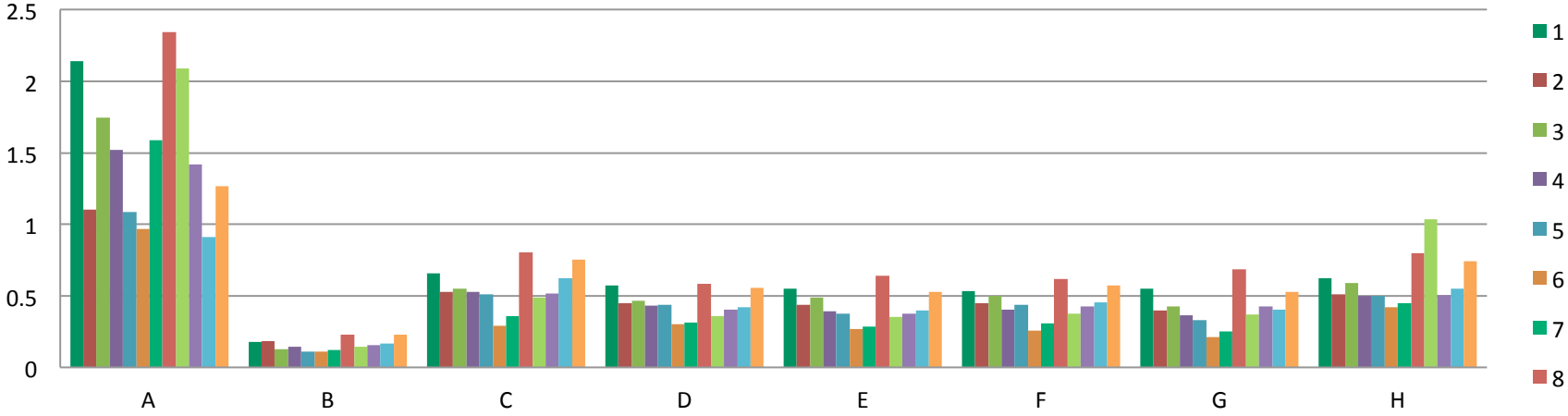
New plate 10 Schizo *vs.* 2 Controls



New plate 10 Schizo *vs.* 2 Controls



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New customized Biolog tryptophan plate
10 Schizophrenia patients *vs.* 2 Controls

Substrate	Schizo CMS 20844	Schizo CMS 20845	Schizo CMS 20846	Schizo CMS 20847	Schizo CMS 20848	Schizo CMS 20849	Schizo CMS 20850	Schizo CMS 20851	Schizo CMS 20852	Schizo CMS 20853	Control CMS 12225	*p* value (Mann-Whitney test)	Control CMS 12223	*p* value (Mann-Whitney test)
a-D-Glucose	2.142	1.104	1.746	1.519	1.085	0.966	1.586	2.342	2.091	1.42	0.909	**0.00195**	1.268	0.10547
Empty	0.179	0.184	0.127	0.145	0.108	0.112	0.12	0.229	0.142	0.158	0.169	0.10547	0.226	**0.00391**
L-Trptophan	0.66	0.529	0.551	0.528	0.509	0.291	0.36	0.805	0.488	0.515	0.625	0.06445	0.755	**0.00391**
Trp-Gly	0.573	0.449	0.464	0.432	0.44	0.303	0.311	0.586	0.357	0.401	0.42	0.76953	0.555	**0.00977**
Trp-Lys	0.552	0.437	0.487	0.393	0.375	0.268	0.283	0.643	0.355	0.377	0.4	1	0.53	**0.01953**
Trp-Ala	0.536	0.448	0.498	0.402	0.439	0.259	0.308	0.616	0.378	0.425	0.452	0.375	0.57	**0.00586**
Trp-Arg	0.549	0.399	0.426	0.362	0.329	0.21	0.249	0.688	0.37	0.424	0.405	0.55664	0.526	**0.01953**
Trp-Leu	0.624	0.512	0.59	0.497	0.498	0.421	0.451	0.796	1.034	0.504	0.553	0.92188	0.742	**0.01953**

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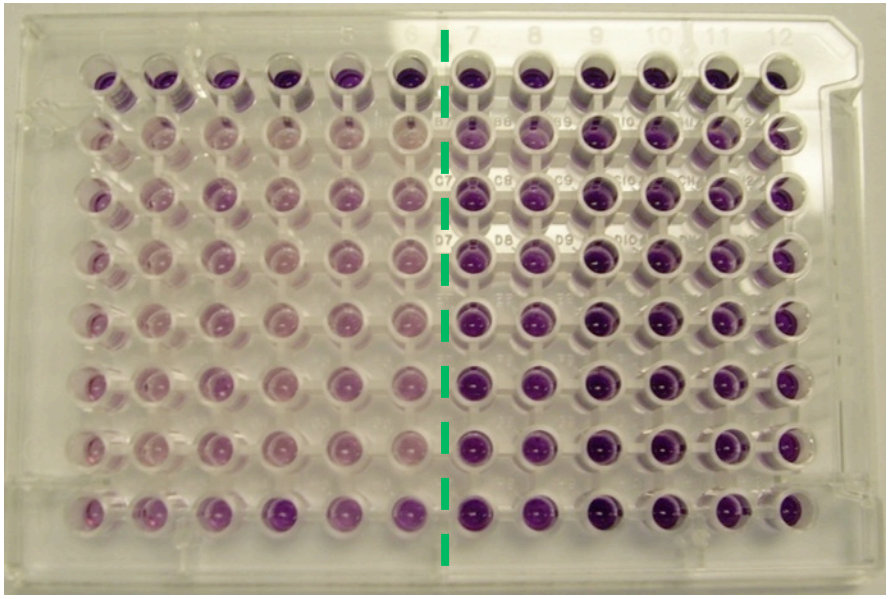

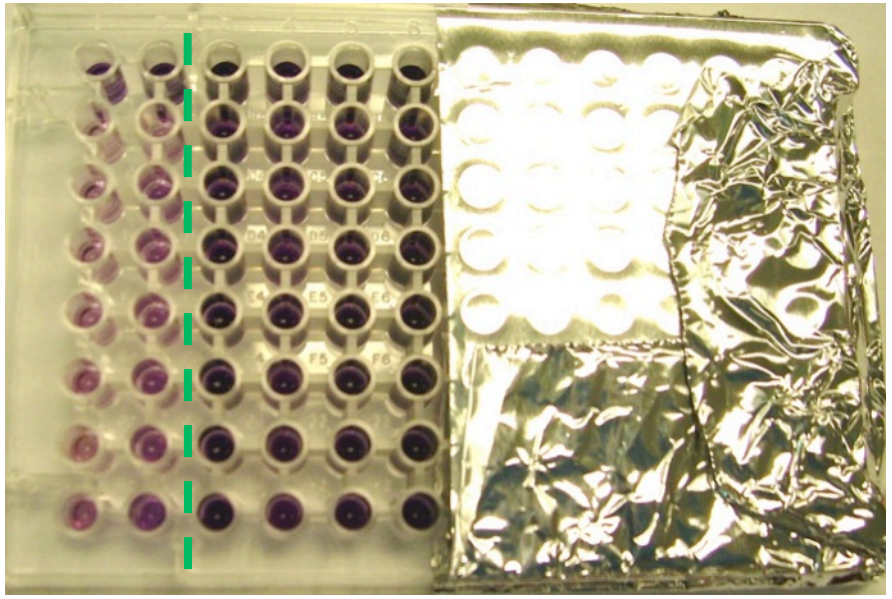

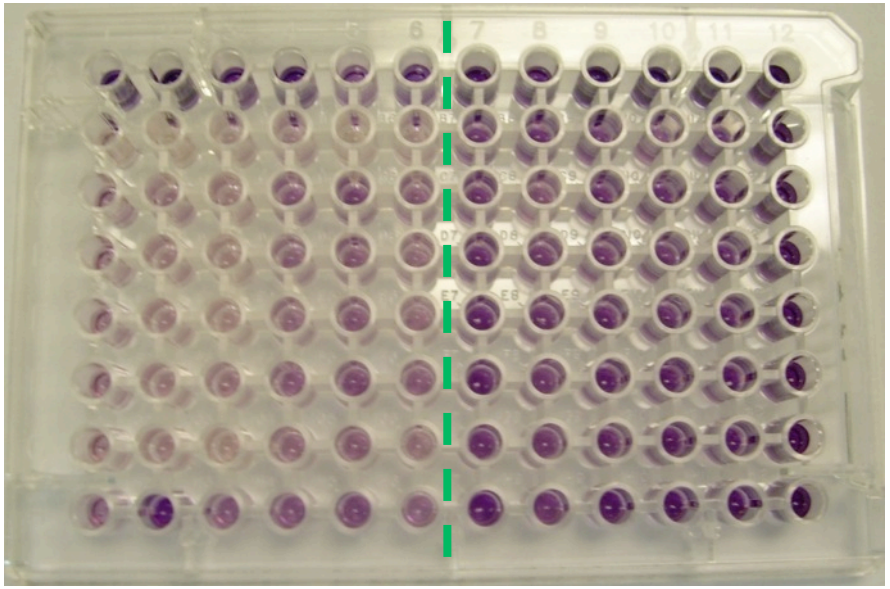

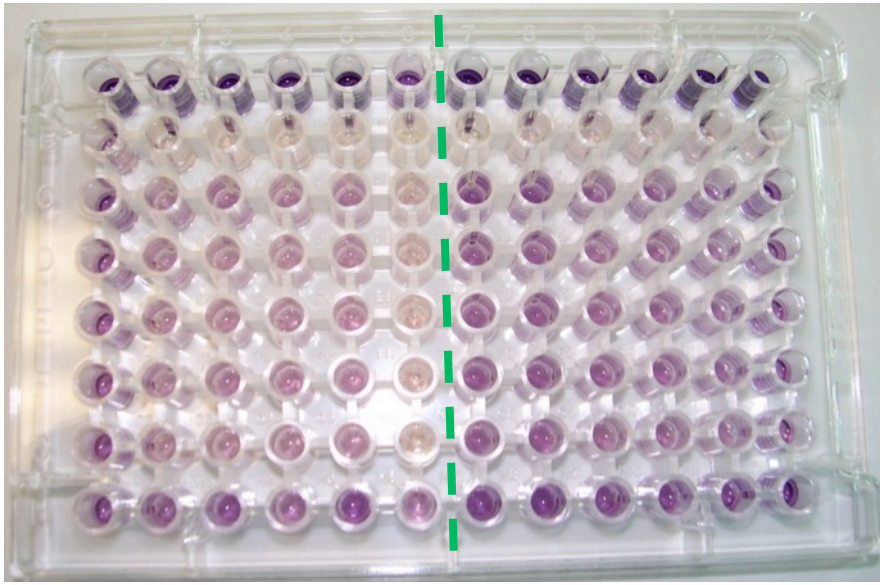

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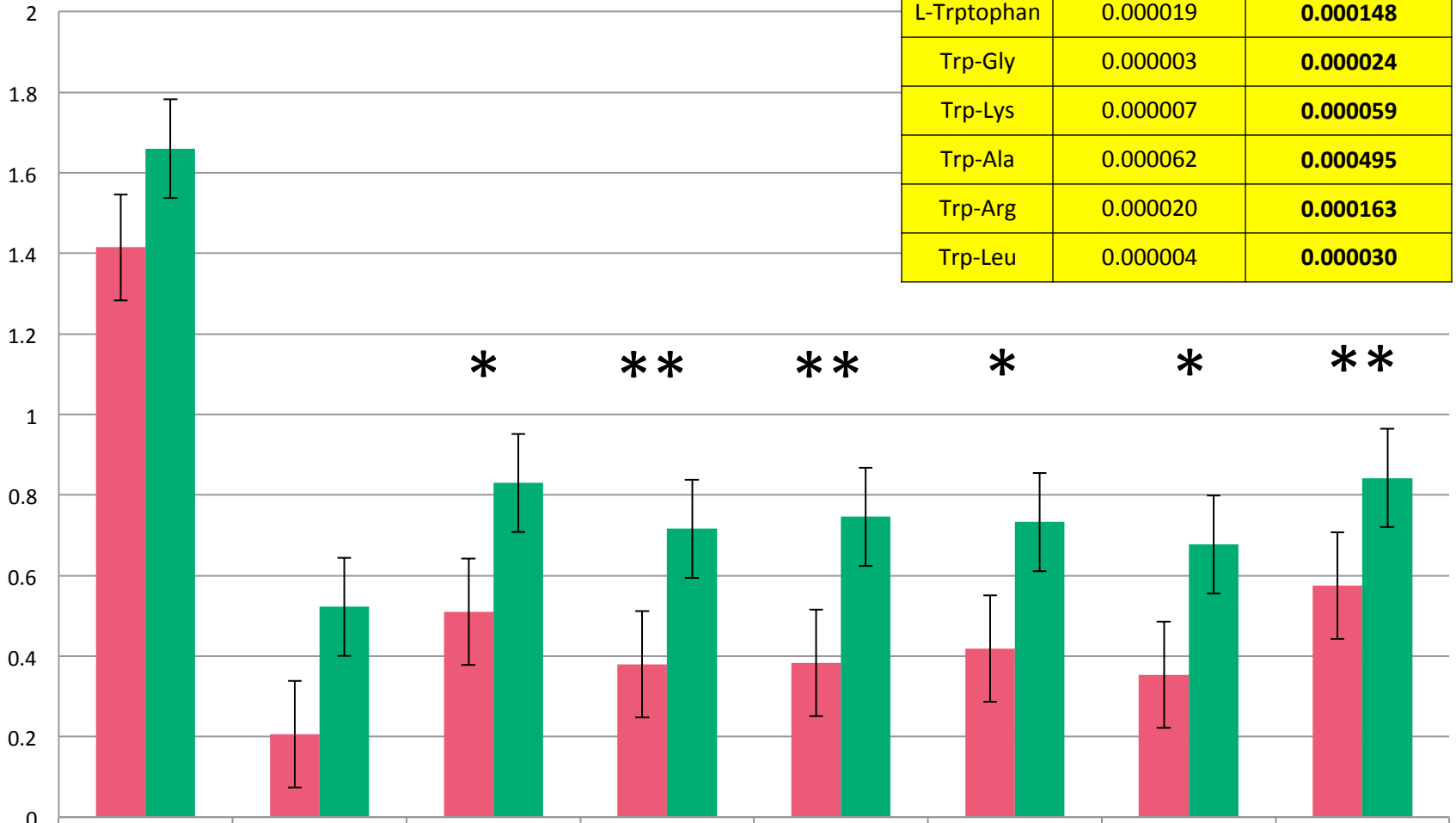
Biolog 50 ASD vs. 50 control cell lines

Substrate	Mann-Whitney *P* value	Bonferroni correction *P* value
a-D-Glucose	0.001344	**0.010756**
Empty	0.023102	**0.184815**
L-Trptophan	0.000019	**0.000148**
Trp-Gly	0.000003	**0.000024**
Trp-Lys	0.000007	**0.000059**
Trp-Ala	0.000062	**0.000495**
Trp-Arg	0.000020	**0.000163**
Trp-Leu	0.000004	**0.000030**

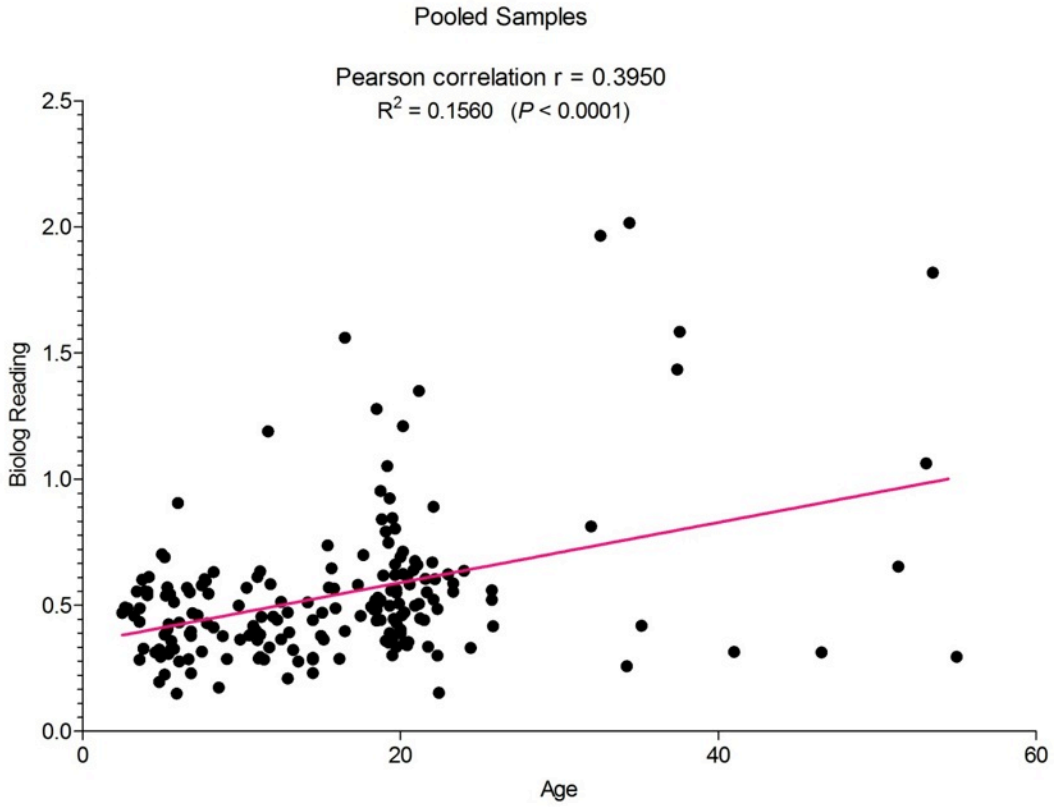
* *P* value <0.001, ** *P* value <0.0001

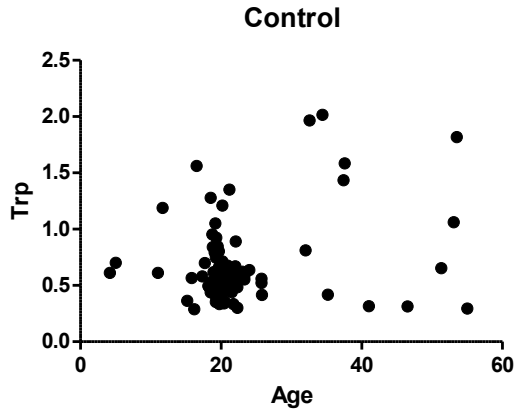
Average ($A_{590-750}$)

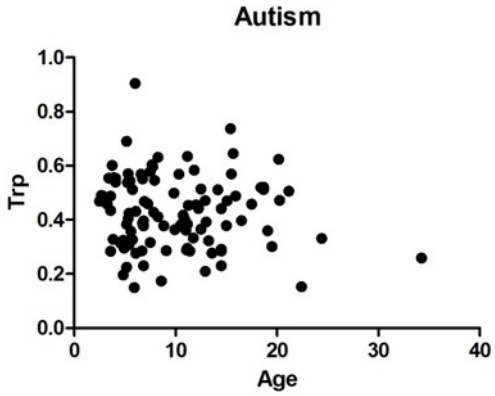


	a-D-Glucose	Empty	L-Trptophan	Trp-Gly	Trp-Lys	Trp-Ala	Trp-Arg	Trp-Leu
50 ASD patients	1.41466	0.20588	0.5102	0.3798	0.38296	0.41872	0.35394	0.57458
50 controls	1.65958	0.52252	0.83004	0.71614	0.74584	0.7328	0.6774	0.84218

Correlation Analysis of the Trp BioLog Data







Age is <u>not</u> influencing the Trp-NADH findings observed in the Biolog arrays.

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Conclusions

✓ NADH generation in the presence of tryptophan as only energy source appears to be significantly lower in lymphoblasts from 87 patients compared to 70 normal controls

✓ This finding was confirmed in both syndromic and non-syndromic cases and is not related to specific genetic alterations

✓ Cell lines from patients with ID and/or behavioral problems, but without autistic traits did not show any difference in NADH production in the presence of tryptophan

✓ Tryptophan metabolites (serotonin, melatonin, kynurenine) have already been linked to behavioral problems and neuronal abnormalities

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Biolog customized tryptophan plate – fresh blood 24 h incubation + 24 h dye

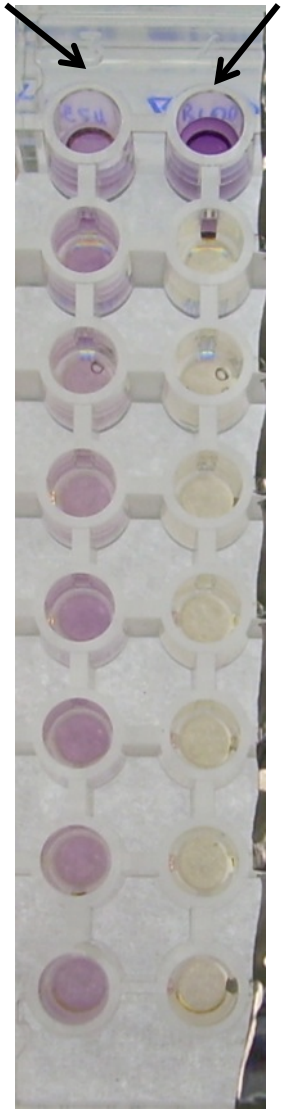
Patient 1

Patient 2

Control 1 (4x)

Control 2*

Patient 3*



* Experiment performed in blinded fashion

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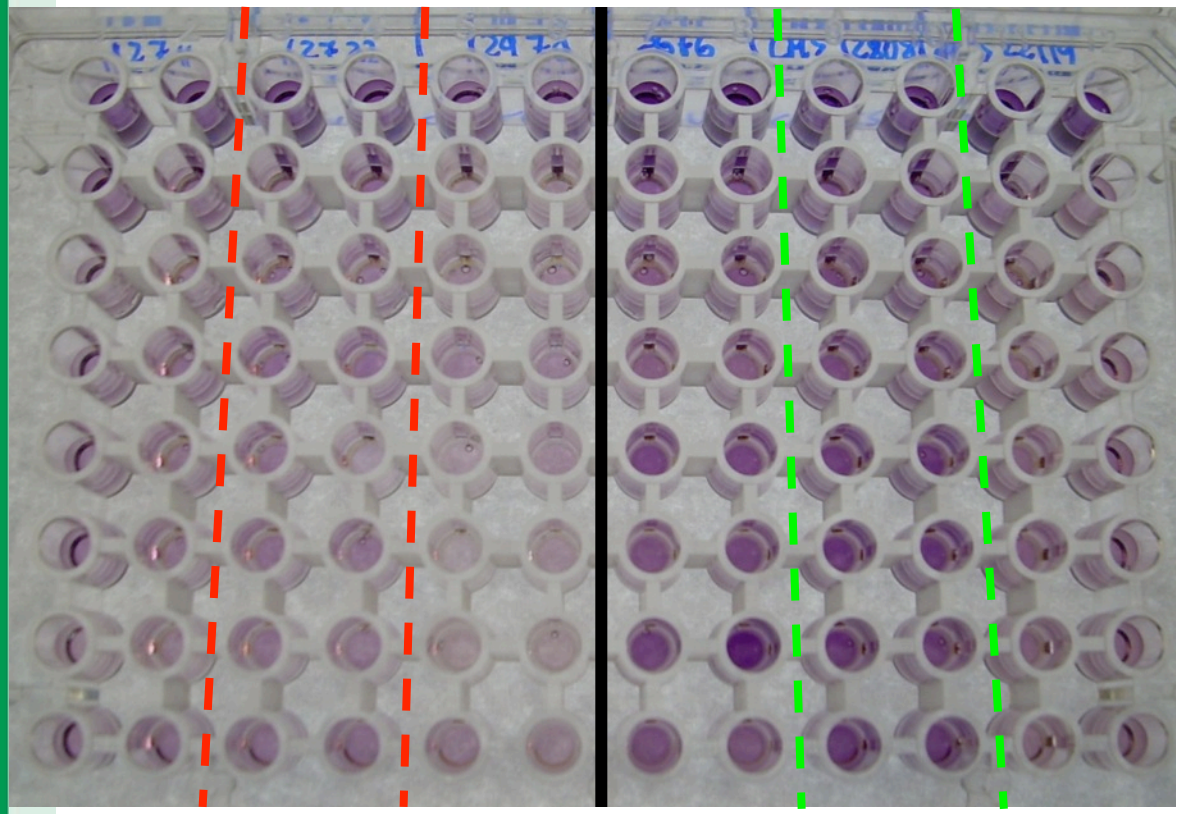
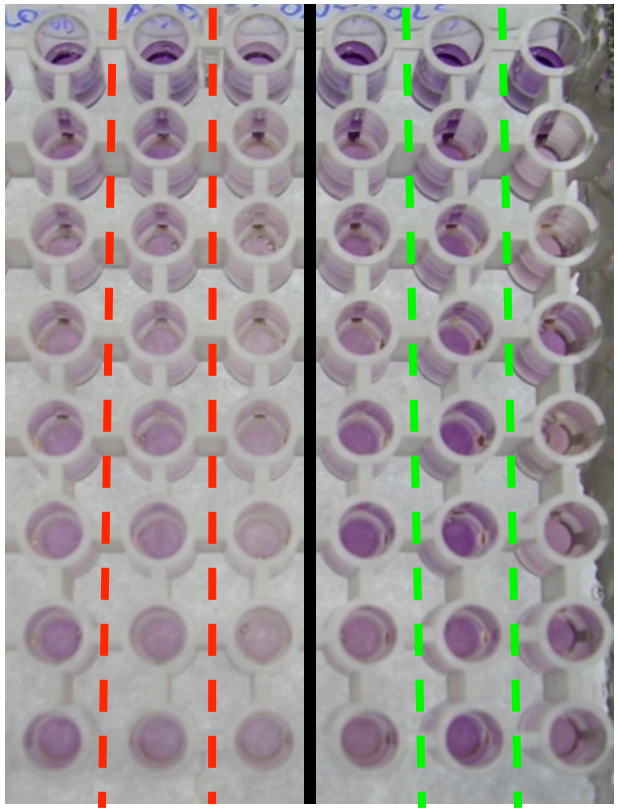
3 SCAP ASD patients *vs.* 3 controls from previous lymphoblastoid cohort (triplicates)





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First 3 ASD patients *vs.*3 controls (previously tested)

Substrate	C1-1	C1-2	C1-3	C2-1	C2-2	C2-3	C3-1	C3-2	C3-3	Control AVG	ASD1	ASD2	ASD3	*P*_value ASD1	*P*_value ASD2	*P*_value ASD3
a-D-Glucose	1.191	1.100	0.947	0.927	0.928	0.953	2.077	1.689	1.494	1.256	0.652	0.724	0.483	0.00391	0.00391	0.00391
Empty	0.497	0.570	0.425	0.550	0.541	0.614	0.669	0.612	0.813	0.588	0.394	0.383	0.209	0.00391	0.00391	0.00391
L-Trptophan	0.512	0.550	0.492	0.568	0.567	0.542	0.742	0.776	0.451	0.578	0.254	0.282	0.212	0.00391	0.00391	0.00391
Trp-Gly	0.449	0.457	0.393	0.594	0.539	0.546	0.700	0.730	0.700	0.568	0.280	0.302	0.223	0.00909	0.00909	0.00909
Trp-Lys	0.669	0.659	0.511	0.689	0.733	0.698	0.756	0.687	0.936	0.704	0.273	0.236	0.182	0.00391	0.00391	0.00391
Trp-Ala	0.501	0.575	0.617	0.747	0.703	0.684	0.799	0.894	0.994	0.724	0.283	0.273	0.148	0.00391	0.00391	0.00391
Trp-Arg	0.633	1.025	0.381	0.700	0.600	0.485	0.688	0.532	0.591	0.626	0.247	0.244	0.146	0.00391	0.00391	0.00391
Trp-Leu	0.466	0.438	0.383	0.678	0.642	0.578	0.753	0.652	0.843	0.604	0.287	0.268	0.199	0.00391	0.00391	0.00391

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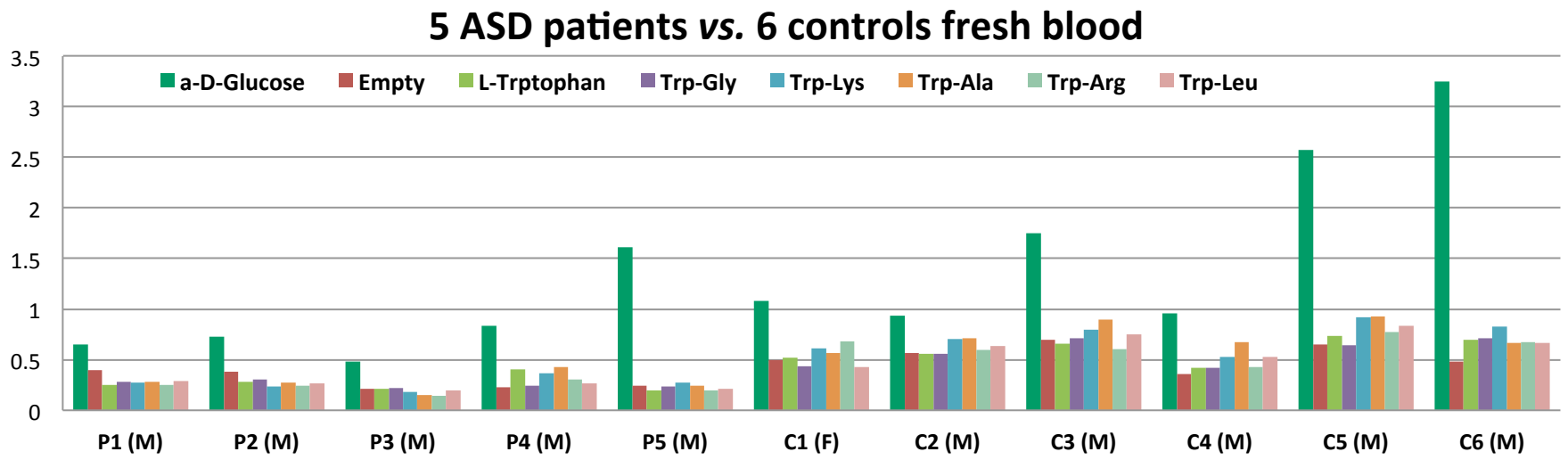
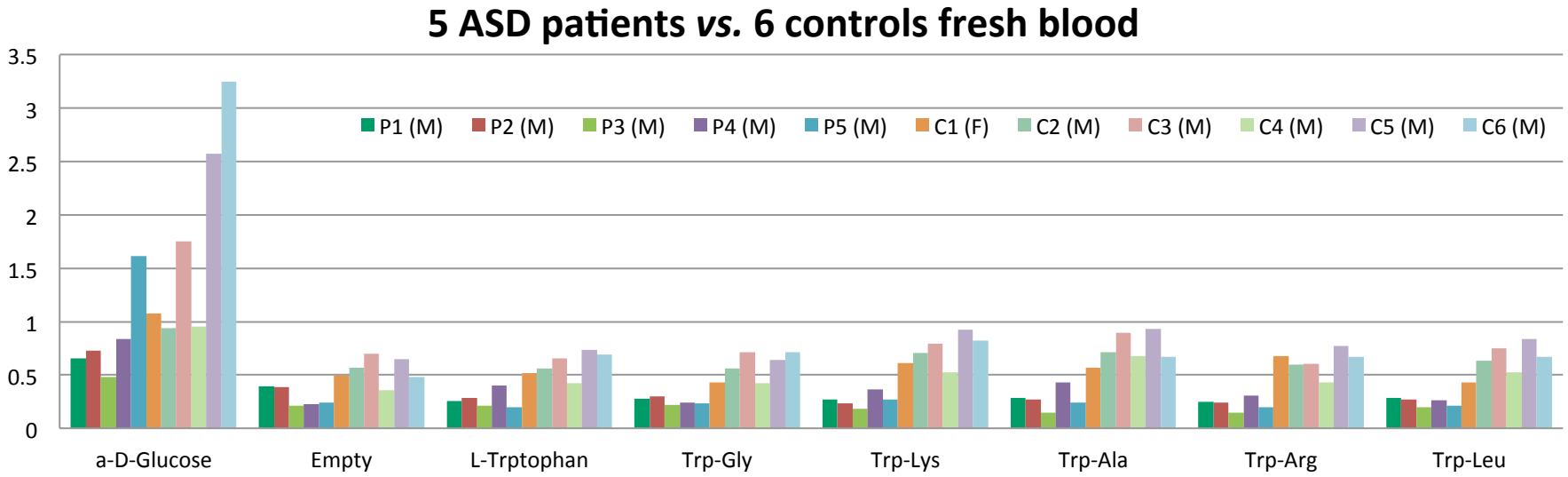
ASD patients *vs.* controls fresh blood

5 ASD patients *vs.*6 controls (previously tested)





ASD patients *vs.* controls fresh blood

12 ASD patients *vs.*12 controls

(not previously tested as cell lines, blinded fashion)

Substrate	Control Average	ASD 1	ASD 2	ASD 3	ASD 4	ASD 5	ASD 6	ASD 7	ASD 8	ASD 9	ASD 10	ASD 11	ASD 12	ASD Average	*P* value (Mann-Whitney)	Comment
a-D-Glucose	2.045	0.756	0.952	0.544	2.630	1.604	2.296	1.016	1.100	0.861	2.717	2.913	1.568	1.580	0.091871	Not significant
Empty	0.498	0.035	0.559	0.372	0.264	0.263	0.426	0.214	0.284	0.088	0.303	0.487	0.108	0.283	0.001227	Lower
L-Trptophan	0.490	0.061	0.439	0.217	0.321	0.241	0.351	0.209	0.268	0.077	0.323	0.453	0.192	0.263	**0.000016**	**Lower**
Trp-Gly	0.535	0.046	0.418	0.253	0.379	0.259	0.345	0.215	0.293	0.086	0.350	0.524	0.186	0.279	**0.000038**	**Lower**
Trp-Lys	0.588	0.051	0.561	0.376	0.392	0.288	0.446	0.126	0.323	0.393	0.359	0.598	0.262	0.348	**0.000214**	**Lower**
Trp-Ala	0.550	0.056	0.556	0.308	0.338	0.267	0.468	0.211	0.294	0.119	0.320	0.536	0.250	0.310	**0.000746**	**Lower**
Trp-Arg	0.496	0.045	0.431	0.262	0.307	0.246	0.285	0.205	0.283	0.075	0.337	0.463	0.258	0.266	**0.000038**	**Lower**
Trp-Leu	0.499	0.063	0.531	0.296	0.332	0.242	0.411	0.207	0.293	0.095	0.320	0.459	0.291	0.295	**0.000368**	**Lower**

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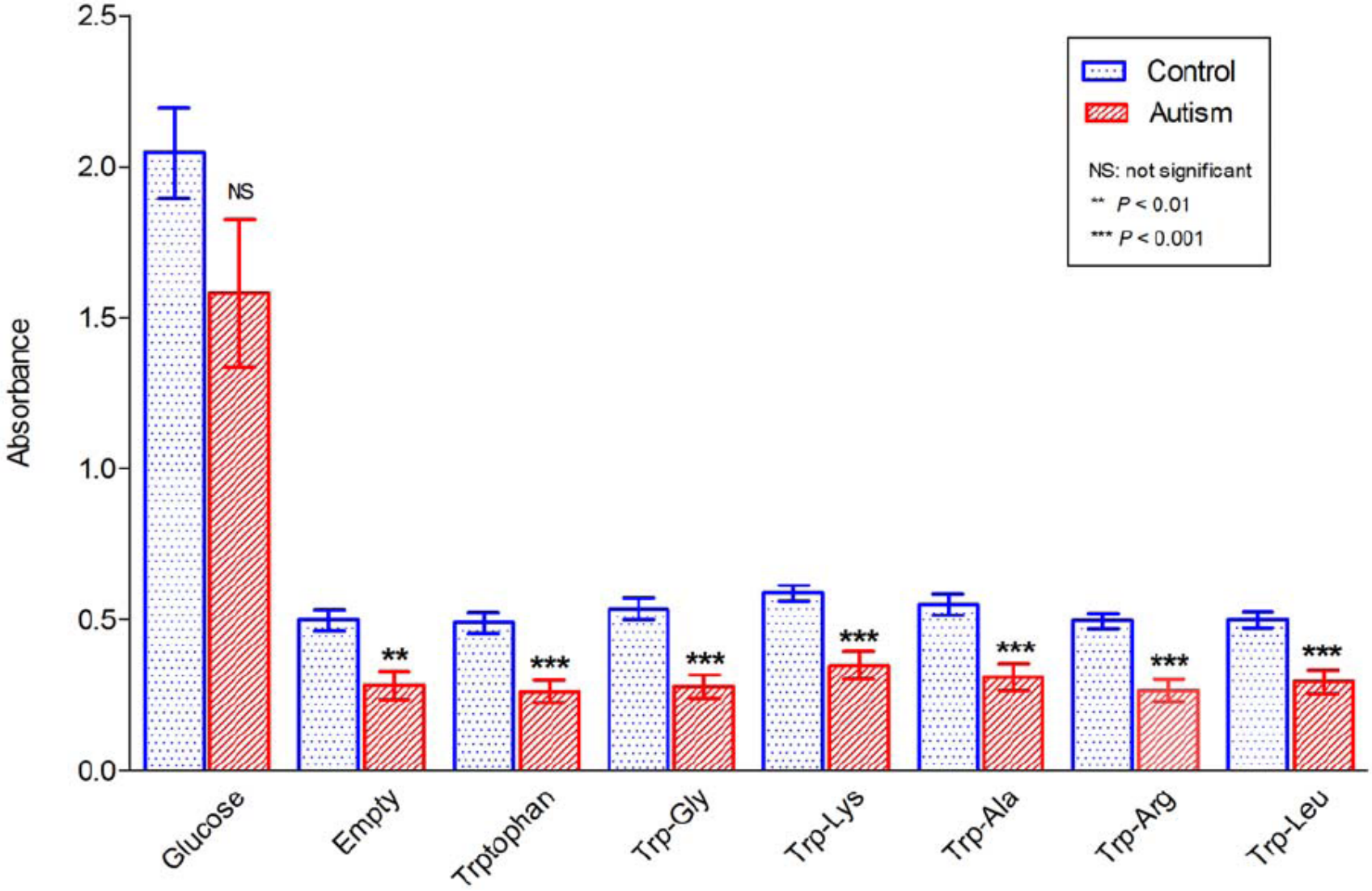
Blood cells BioLog results (12 vs. 12)



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Tryptophan transporters

Transporter	Locus	Linkage to ASDs	Amino acid	Expressed in blood	Expressed in brain
SLC16A10	6q21-q22	No	Aromatic	No	No
SLC7A5	***16q24.3***	**No**	***Neutral***	***YES***	***YES***
SLC7A8	***14q11.2***	**No**	***Neutral***	***YES***	***YES***
SLC3A2	***11q13***	**Yes (Schellenberg *et al* Mol Psychiatr 2006)**	***Neutral***	***YES***	***YES***
SLC6A19	5p15.33	Yes (Sztamari *et al* Nat Genet 2007)	Neutral	No	No
SLC6A14	Xq23-q24	Yes (Liu *et al* Am J Hum Genet 2001; Jacquemont *et al* J Med Genet 2006)	Neutral	No	No

The Trp transport depends on the Large neutral Amino acids Transporter (LAT), a multimeric unit composed by a **heavy subunit** protein**, 4F2hc (*SLC3A2*)**, and 2 possible **light subunit** proteins: **CD98 (*SLC7A5*)**, that will give LAT1, and the **Large neutral amino acids transporter small subunit 2 (*SLC7A8*)**, that will give LAT2.

SLC3A2 variants (107 ASD patients)

Patient	Variant	Effect/ Bioinformatics	Parents/controls	SNP (1000 genomes/NHLBI)
17550 (BM)	c.6 G>A (Exon 1)	p.Glu2Glu; 4 new ESEs; no qPCR changes	Mother: negative; 2/274 (0.7%) controls vs 1/107 patients (0.9%)	rs114958414 G: 2183 (99.96%); A: 1 (0.04%) NHLBI G: 12994 (99.96%); A: 6 (0.04%)
12382 (WM)[1]	c.826 G>A (Exon 5)	p.Asp276Asn Benign	Father; 0/281 controls	rs80190679 (G: 99.9%; A: 0.1%) NHLBI G: 12981 (99.9%); A: 17 (0.1%)
	c.902-24 T>A (Intron 5)	None	Negative (*de novo*)	No
18454 (BM) [2]	c.1027 G>T (Exon 7)	p.Asp343Tyr Deleterious (6/8 websites)-Splice changes	No parents available; 0/542 controls *vs.* 1/107 patients (0.9%)	No
17410 (WM) [3]	c.1352 G>A (Exon 10)	p.Arg451Gln Deleterious (5/8 websites)	Father (affected brother: Negative)	rs148443520 G: 10755 (99.97%); A: 3 (0.03%) NHLBI G: 12995 (99.98%); A: 3 (0.02%)
17843 (WM)	c.1316 G>T (Exon 10)	p.Arg439Met - Borderline (4/8 websites)	Father (mother not available)	rs116829615 (G: 99.7%; T: 0.3%) NHLBI G: 12979 (99.9%); T: 19 (0.1%)
15294 (BF)	c.594 T>G (Exon 4)	p.Ala198Ala	Not tested	No
17226 [4] - 9754 - 18454 [2] (BM)	c.1606 C>T (Exon 12)	p.Leu536Leu	17226: mother and unaffected twin sister= Negative	rs76688902 (C: 98.1%; T: 1.9%) NHLBIG (C: 97.5%; T: 2.5%)
17817-14037-12970 -12053 (BM)	c.902-11 C>T (Intron 5)	Higher Signal Quantile (69->78) at Acceptor Splice Site	12970: both parents are heterozygous	rs59762129 (C: 95.5%; T: 4.5%)

[1] del (20)(p11.22;p11.23), [2] *EGR2* SNPs, [3] *MET* pol C/C, [4] *FRAXE* 0 repeats

SLC7A5 variants (107 ASD patients)				
Patient	**Variant**	**Effect/Bioinformatics**	**Parents/controls**	**SNP (1000 genomes/NHLBI)**
10471 (HM)[1]	**c.1123 C>A (Exon 7)**	**p.Pro375Thr – Deleterious**	**Mother and relatives: negative; 0/542 controls *vs.* 1/57 patients (1.75%), *p* value: 0.0952**	**No**
	c.1347 C>G (Exon 9)	**p.Val449Val**		**rs11541881 C: 2166 (99.2%); G: 18 (0.8%) NHLBI C: 12969 (99.98%); G: 3 (0.02%)**
CMS 14985 (WM-PDD)	**c.1141-98 T>A (Intron 7)**	**Acceptor splice site changes**	**No parents available**	**No**
15301 (BM)	**c.1290+44 G>A (Intron 8)**	**Lost 1 ESE**	**Mother: negative**	**No**
CMS 15811 (WM)[2]	**c.816-18 C>T (Intron 4)**	**Higher Signal Quantile (57 -> 59) at Acceptor Splice Site**	**No parents available**	**No**
14988 (BM)	**c.1141-93 C>T (Intron 7)**	**None**	**Not tested**	**No**
4 patients (1 Hom)	**c.690 C>G (Exon 3)**	**p.Asn230Lys Lost 2 ESEs**	**Father (12970); 10/282 controls (3.5%)**	**rs1060250 (C: 98.7%, G: 1.3%) NHLBI C: 12768 (98.3%); G: 228 (1.7%)**
12970 (BM) [3]	c.387 C>T (Exon 1)	p.Ala129Ala	Father	rs33913122 (C: 99.8%, T: 0.2%) NHLBI (12966) C: 99.97%, T: 0.03%
12 patients, 11.2%	**c.843 C>T (Exon 5)**	**p.Ser281Ser**	**Not tested**	**rs33992288 (C: 99.8%; T: 0.2%) NHLBI (12994) C: 99.95%, T: 0.5%**
9754 (BM)	c.939+20 C>T (Intron 5)	None	Not tested	rs33993343 (C: 99.8%; T: 0.2%)
9754-14988 (BM)	c.1008 C>T (Exon 6)	p.Phe336Phe	Not tested	rs1060251 (C: 99.2%; T: 0.8%) NHLBI (12996) C: 98.9%, T: 1.1%
12388 (BF)-17226 (BM)[4]	c.1043+6 C>T (Int 6)	Higher Splice Score (10.1 -> 12.2) at Donor Splice Site	Not tested	rs33966404 (C: 98%; T: 2%)

[1] *ZNF711* mut; [2] *FMR1* full mutation; [3] 47,XY +del(15)(q14); [4] *FRAXE* 0 repeats

SLC7A8 variants (107 ASD patients)				
Patient	**Variant**	**Effect/Bioinformatics**	**Parents/controls**	**SNP (1000 genomes/NHLBI)**
12388 (BF)	**c.47 C>G (Exon 1)**	**p.Pro16Arg – Borderline (4/8 websites)**	**Mother and affected brother**	**rs147920363 C: 2180 (99.8%); G: 4 (0.2%) NHLBI C: 12964 (99.7%); G: 42 (0.3%)**
6320 (WM)[1] 17673 (BM)	**c.52 G>T (Exon 1)**	**p.Gly18Trp – Deleterious Decreased Signal Quantile (19->8) at Donor Splice Site**	**Negative (6320-*de novo*; 17673-Mother); 2/542 controls vs 1/107 patients (0.36% vs 0.9%,)**	**rs144958980 G: 2175 (99.6%); T: 9 (0.4%) NHLBI G: 12930 (99.4%); T: 76 (0.6%)**
4947 (WM)	**c.86 C>T (Exon 1)**	**p.Ser29Phe – Deleterious (5/9 websites)**	**No parents available; 2/542 controls vs 1/57 patients (0.36% vs 0.9)**	**rs149980964 C: 2181 (99.9%); T: 3 (0.1%) NHLBI C: 12970 (99.8%); T: 36 (0.2%)**
15318 (WM) [1]	**c.1016-49 T>C (Intron 7)**	**None**	**Negative (*de novo*)**	**No**
CMS 6276 (WM) [2]	c.1125 C>T (Exon 9)	p.Thr375Thr	No parents available	rs141169165 (C: 99.9%; T: 0.1%) NHLBI (13006) C: 99.92%, T: 0.08%
CMS 1836 (WF) [3]	c.1113+19 G>A (Intron 8)	None	No parents available	No
23 patients, 21.5% (2 Hom, 1.9%)	**c.120 C>T (Exon 1)**	**p.Ile40Ile, 2 new ESEs**	**Not tested**	**rs1884545 (C: 94%; T: 6%) NHLBI (13006) C: 89.6%, T: 10.4%**
12713 (WM)	**c.634+10 G>T (Intron 4)**	**1 new ESE**	**Father**	**c.634+10 G>A is SNP rs35329117 (no frequency data, no ESE change)**
20 patients, 18.7% (5 Hom, 4.6%)	**c.635-83 T>A (Int 4) [4]**	**1 new ESE**	**Not tested**	**rs10151002 (T: 93%; A: 7%). Genotypes (1092): T/T: 87.8%, T/A: 10.4%, A/A: 1.7% NHLBI (13006 alleles) T: 99.992%, A: 0.008% (1)**
66 patients (16 Hom)	c.913-13 G>T (Intron 6)	Increased Signal Quantile (42 ->63) at Acceptor Splice Site	Not tested	rs1015089 (G: 65.1%; T: 34.9%)
89 patients, 83.1% (31 Hom, 29%)	c.1170 T>C (Exon 9)	p.Tyr390Tyr, 1 new ESE	Not tested	rs7157021 (T: 33.7%; C: 66.3%) NHLBI (13006): T: 41.3%, C: 58.7%

[1] *ZBTB20* mut ; [2] *FMR1* full mutation ; [3] *MECP2* (c.502 C>T; p.R168X); [4] T>A genomic, A>T cDNA

Fragile X syndrome

- Moderate to severe **ID**, **macroorchidism**, and distinct **facial features**, including long face, large ears, and prominent jaw.
- About 1/3 of the patients show **autistic traits**, other **behavioral issues** (i.e.: ADD/ADHD, anxiety/OCD) are also common.
- First monogenic cause of ID (1/4,000 males).
- Unstable **expansion of a CGG repeat** in the *FMR1* promoter and abnormal methylation → **suppression of FMR1 transcription** and decreased protein levels in the brain.

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Preliminary data – Fragile X syndrome

12 cases (8 with full mutation, 4 female carriers) versus 12 controls

Considering just the Fragile X diagnosis, neither the patient nor the carrier cohort showed significant metabolic differences as compared to controls

Two subgroups with mental disorders:

- **3** cases with **ADD/ADHD** (and **Anxiety/OCD**)
- **6** cases with **Anxiety/OCD** (including the 3 with **ADD/ADHD**)

In both subgroups we noticed a statistically significant **increase of utilization of glucose-related sugars, Krebs cycle intermediates, and ketone bodies,** as compared to controls.

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Preliminary data - Fragile X patients with ADD/ ADHD and Anxiety/OCD (3)

Significantly increased utilization of:

- **glucose-related sugars** (14/27, 51.8%), particularly glucose*
- **n-acetyl-neuraminic acid:** predominant sialic acid in gangliosides (crucial component of neuronal membranes)
- **pyrimidines** (2/2): thymidine and uridine*
- **Krebs cycle intermediates** (7/9, 77.7%): particularly lactate*, succinate*, and malate*
- **ketone bodies and other carboxylic acids** (9/13, 69.2%): particularly acetoacetate*, b-hydroxy-butyrate*, propionate*, and acetate*
- most **amino acids**: some (Asn*, His*, Met*, and Val*) were significant for all the wells in the arrays.

* Significant in 3/3 cases

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Preliminary data – Fragile X patients with Anxiety/OCD (6)

Significantly increased utilization of:

- **glucose-related sugars** (9/27, 33.3%), particularly glucose*
- **n-acetyl-neuraminic acid**
- **pyrimidines** (2/2): thymidine and uridine*
- **Krebs cycle intermediates** (6/9, 66.7%): particularly lactate* and pyruvate*
- **ketone bodies and other carboxylic acids** (6/13, 43.1%): particularly acetoacetate*, b-hydroxy-butyrate*, and acetate*
- Only Asn* was significant for 80% of the wells in the arrays.

* Significant in at least 4/6 cases

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Background (ID and ADD/ADHD)

- **Neuronal cells** have one of the **highest metabolic rates** in the human body and use sugars and ketone bodies as primary energy sources.

- Abnormal metabolism of sugars and ketone bodies may affect **neuronal development, signal transmission and synaptic function.**

- Several mental disorders have been associated with **increased metabolism** within specific brain areas as detected by PET studies.

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Hypothesis (ID and ADD/ADHD)

- Some mental disorders have a **late onset** (ADD/ ADHD, OCD, Tourette syndrome) and can occur after an **event causing an increase in metabolic rate** (high fever, infections, brain damages, puberty).

- When compared to controls, cells from patients with **ID and developmental mental disorders like ADD/ ADHD** appear to have **increased need of energy sources** under situations promoting **metabolic stress** and the consequences can be more severe in those tissues with higher metabolic rates (i.e. neuronal cells).

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Summary

- Biolog phenotype microarray plates are able to identify **significant abnormalities in the metabolic profile** of cells from patients with **neurodevelopmental disorders**, like ASDs, ADD/ADHD, and anxiety/OCD.
- The metabolic findings reveal **new molecular pathways** playing an important role in the **pathogenesis** of those disorders and can provide **new tools for therapeutic approaches**.
- Follow-up studies involving **kinetics and gene expression analysis** are ongoing to better characterize these findings.

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