Tyrosine Hydroxylase Deficiency: Studies in patient samples and in a cellular model

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Memòria presentada per Alba Tristán Noguero per optar al títol de doctor per la Universitat de Barcelona

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Barcelona, ……

Ningú ens ha dit que el temps
no deixa cap ferida;
però pels camins tranquils
hi ha poca poesia…

Per tu Paparoti,

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ABSTRACT

Monoamine Neurotransmitter diseases are a rare group of inherited disorders of metabolism that encompass 12 different genetic defects leading to abnormal dopamine and/or serotonin brain homeostasis. They correspond to enzymatic deficiencies involved in the biosynthesis, catabolism and transport of dopamine and serotonine. From a clinical point of view, they can appear at any age and manifest diverse clinical features. However, movement disorders ranging from dopa-responsive dystonia to severe parkinsonism, associated to variable degrees of cognitive impairment is the most common form of presentation.

The pathophysiology underlying the wide spectrum of clinical phenotypes (from mild to severe) and response to neurotransmitter precursors (L-Dopa+carbidopa, BH4 and other dopaminergic enhancers) has not been studied in detail. Here we aim to address this important issue through different approaches: i) the study of a particular disease of neurotransmitters, Tyrosine Hydroxylase deficiency (THD), as a model of dopaminergic deficiency. Tyrosine hydroxylase (TH) enzyme catalyses the rate-limiting step in the biosynthesis of dopamine (DA). THD exhibits a wide spectrum of clinical manifestations that have been grouped according to the severity in two clinical phenotypes: “Type A” tends to present as L-Dopa responsive parkinsonism-dystonia whereas “Type B” produces a severe encephalopathy of early-onset with sub-optimal L-Dopa response. ii) The study of a large cohort of patients with neurotransmitter defects thanks to an international collaboration (I-NTD group). We have used both patients’ samples and an iPSc model to address these questions.

A) Patients’ samples:

A.1) We studied one THD B phenotype postmortem brain and we observed that the expression of key synaptic proteins and neurodevelopmental markers were altered: TH, VMAT 1 and 2 and dopamine receptors, especially D2DR were decreased. GABAergic and glutamatergic proteins such as GABAVT, NMDAR1 and calbindin were also altered. Finally, developmental markers for synapses, axons and dendrites were decreased, whereas markers of neuronal volume were preserved.

A.2) 94 CSF samples of patients with neurotransmitter defects from 9 centres belonging to different countries/continents were collected in an international collaboration through the I-NTD working group. The proteomic study showed that the main category of overrepresented proteins was related to nervous system development. Moreover, different proteins were detected that could be useful biomarkers for severity prognosis and response to treatment that are specific of disorders. Four of them were correctly validated with an ELISA analysis: APOD, COL6A3, APOH and OMGP. These proteins are involved in diverse important biological functions such as myelination, phospholipid and other lipid related processes.

B) iPSC model of THD:

iPSC lines from Type A and B patients, controls and an isogenic corrected iPSC line were generated. Upon Dopaminergic differentiation, THD A and B neurons reproduced the disease-associated phenotype: decreased TH Protein, reduced enzyme activity and alteration on DA genes expression. A new neuronal phenotype was also described: less TH-immunoreactive cells and fiber density in both mutant TH+ neurons, Type A and B DAn presented altered morphology (reduced neuronal arborisation only in THD-Type B) and a reduced axonal TH localization was observed in THD-Type A. We were also able to test therapeutic approaches such as L-Dopa + carbidopa.

To conclude, we have performed extensive and novel studies using different approaches and techniques to better characterize the pathophysiology underlying the spectrum of severity and response to the current pharmacological treatments of neurotransmitter defects. We have provided new information pointing towards a dysregulation of multiple neurodevelopmental functions in these diseases, and biomarkers of clinical severity that could be explored in the future as therapeutic targets. Additionally, an iPSC model for THD has been developed for the first time that introduces mechanistic and therapeutic insights in this early-parkinsonism model.

TABLE OF CONTENTS

INTRODUCTION

1. Neurotransmitter defects (NTD).

A neurotransmitter is a chemical messenger that carries signals between neurons and/or nerve cells or with other cells in the body. There are more than a hundred of different molecules that can act as a neurotransmitter that belong to different biochemical categories such as amino acids, peptides, monoamines or purines among others.

Classical neurotransmitters include amino acids (GABA, glycine and glutamate), purines, cholinergic transmission and monoamines (Serotonin, dopamine, adrenaline and noradrenaline) (Sheffler and Pillarisetty).

Neurotransmitter defects are a group of inherited metabolic disorders that present with deficiencies in the synthesis, catabolism and transport of different neurotransmitters. (Cortès-Saladelafont et al, 2015). The ones that have been studied and characterized in detail from a clinical point of view and have diagnostic biomarkers are basically the ones that affect amino acids and monoamines. (Hoffmann 2010).

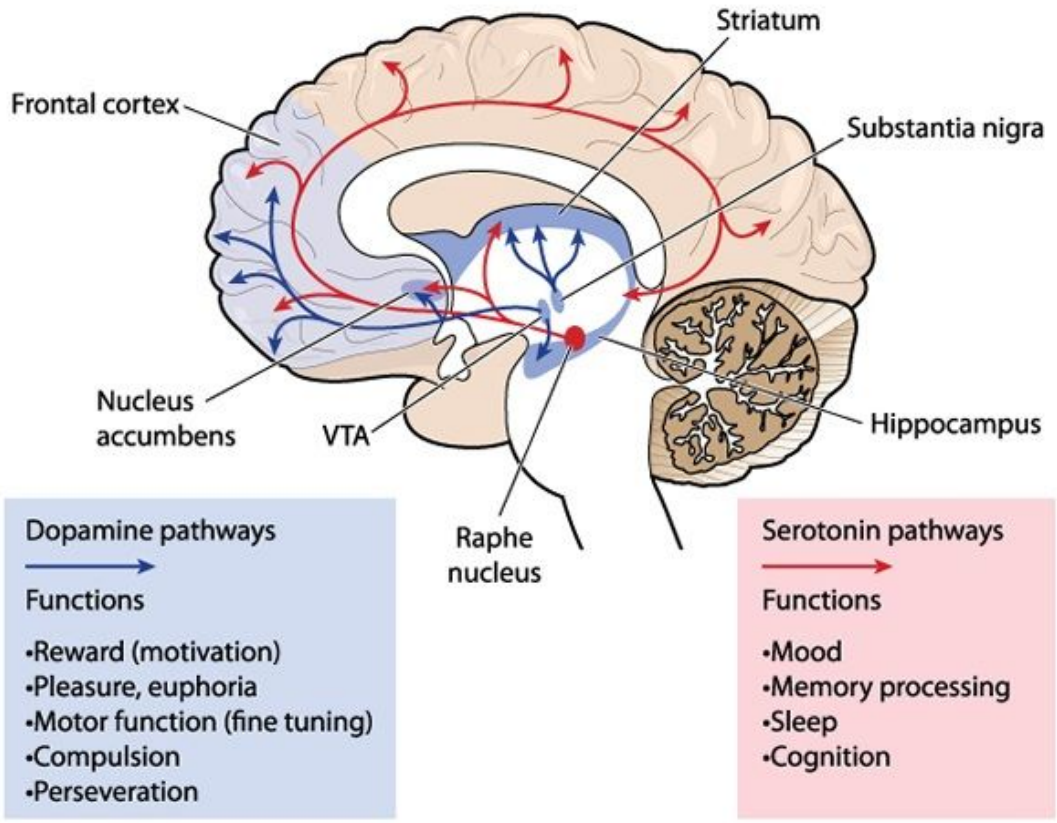
Regarding the physiological functions of monamines, they are diverse depending on each individual molecule. Serotonin modulates different neuropsychological processes and neural activity, gastrointestinal system and cardiovascular function. It also regulates mood, behaviour and sleep, what makes serotonin an important target for different neurological and psychiatric drugs. Dopamine plays an important role in several brain functions including motor control, reward or pleasure. Dopamine has also been implicated in diverse psychiatric and neurological conditions (Sheffler and Pillarisetty).

Monoaminergic innervation has two distinctive pathways:

- serotoninergic pathway: 5-HT (5-hydroxytriptophan) cell bodies, mainly localized in the raphe nuclei, send axons to almost every brain region.

- dopaminergic pathway is more limited in space and, includes two sub-pathways: i) the nigrostriatal pathway that initiates in the Substantia nigra (SN) and innervates the striatum; ii) the mesocorticolimbic pathway that initiates in the Ventral tegmental area (VTA) to innervate the raphe nucleus, the nucleus accumbens (NAc) towards the frontal cortex (Figure 1)

Figure 1: Schematic representation of Serotonin and Dopamine pathways



Genetic mutations that affect the biochemical pathways of monoamines are rare neurological diseases that can appear at any age but are especially known as pediatric conditions. Description of clinical manifestations, genotypes, biomarkers and pathophysiology started in the last two decades and have layed the ground to understand the basic principles of these rare disorders that belong to the category of Inborn Errors of Neurotransmission.

Neurological manifestations of NTDs (focused on monoamines) include movement disorders, developmental delay, intellectual disability and complex encephalopathies but can vary between the different disorders. Moreover, there is a wide spectrum of severity within the same genetic defect ranging from dopa-responsive dystonia to severe encephalopathies.

These disorders can be detected at any age or sex. Most of them are diagnosed by means of cerebrospinal fluid (CSF) biochemical profile, which is characteristic of each disorder and further confirmed by the mutational screening Table 1 shows reference values of monoamine metabolites in the CSF according to different age ranges. Table 2 shows specific CSF biochemical profile for every defect. .

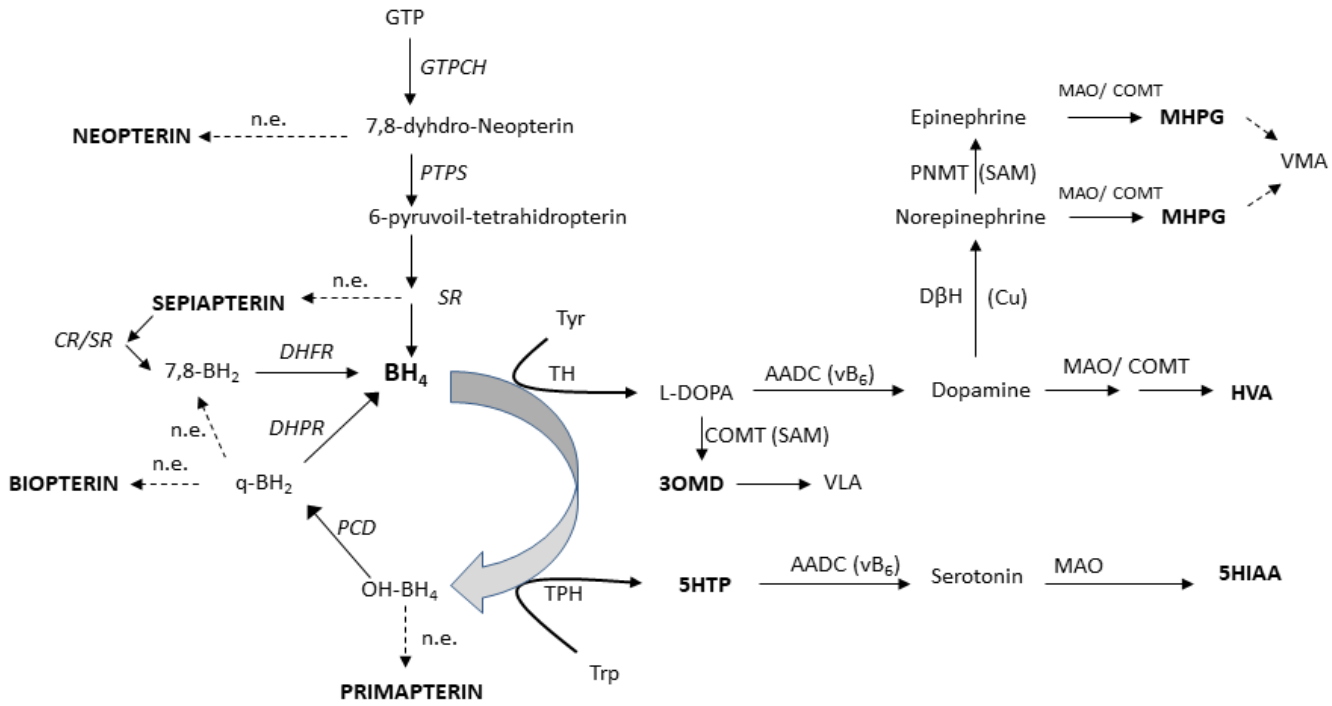
The treatment differs in every genetic defect but as a rule is it based on the supplementation of the deficient neurotransmitter or its pharmacological enhancement (i.e. dopaminergic agonists, monoaminooxidase inhibitors)

Table 1: Reference CSF biochemical values

Age	5-HIAA	HVA	HVA/5-HIAA	3-OMD	MHPG	5-HTP
0 - 30 d	428 – 1122	658 – 1434	0.76 – 1.67	24 – 148	44 – 106	6.0 – 24.0
1 - 5 m	217 – 1142	354 – 1328	1.16 – 2.4	20 – 162	30 – 124	2.7 – 26.0
6 m- 2 y	170 – 490	344 – 906	1.11 – 3.48	4 – 50	20 – 80	1.6 – 15.0
3 - 6 y	106 – 316	304 – 658	1.92 – 3.44	3 – 64	22 – 54	4.0 – 23.0
7 - 10 y	87 – 366	202 – 596	1.20 – 3.45	5 – 60	13 – 68	1.6 – 16.0
11 - 16 y	63 – 185	156 – 410	1.44 – 3.17	3 – 54	11 – 46	2.4 – 12.0

Legend: d: days; m: months; y: years

Figure 2: Biochemical pathways of monoamines and pterins:



1.1 Disorders of monoamines synthesis

1.1.1 BH4 deficiencies

GTP cyclohydrolase (GTPCH) deficiency: GTP cyclohydrolase (GTPCH-I) is the rate-limiting step in the biosynthesis of BH4, an essential cofactor for different amino acid hydroxylases, being the Tyrosine Hydroxylase the one that presents higher affinity. Deficiency of GTPCH can be present in a dominant (Segawa disease) or a recessive form and produces DRD, parkinsonism and Intellectual disability in the recessive form. GTPCH deficiency can be diagnosed by low levels of HVA, pterines and 5-HIAA (can be normal in the dominant form) in the CSF. Patients have been treated with a combination of low dose L-dopa (4 to 5 mg/kg/day) and a dopa-decarboxylase inhibitor. There is normally a complete or near-complete response of motor problems soon after the start of the therapy. Even when the therapy is started after a diagnostic delay of several years, the results are satisfactory. However, in cases of action dystonia and adult onset cases, levodopa does not al-ways show complete effects. The recessive form may mimic THD phenotypes regarding L-Dopa response (Opladen et al. 2011).

Sepiapterin reductase (SRD) deficiency: Sepiapterin reductase catalyses the last two steps in the BH4 synthesis. Sepiapterin reductase deficiency is an autosomal recessive disorder that produces dystonia responsive to L-Dopa (DRD), Parkinsonism, Oculogyric crises and intellectual disability.

The biochemical profile of the CSF comprises low levels of HVA and 5-HIAA and increased levels of biopterin and sepiapterin. For SRD, therapeutic approaches involve dopamine and serotonin precursor supplementation, and most patients respond well to L-dopa and 5-hydroxytryptophan combination. As regards dopaminergic disturbances, improvement in motor and sleep symptoms have been reported with the combination of L-dopa and carbidopa. Since dyskinesias may appear after treatment, a very low starting dosage of L-dopa (around 0.5 mg/kg per day) with slow increment is advised. Regarding 5-HTP treatment (serotonin precursor), improvement in sleep, motor and cognitive aspects have been reported (doses ranging from 1 to 6 mg/kg per day) (Bonafé et al. 2001).

Pyruvoyltetrahydropterin synthase (PTPS) deficiency: 6-pyruvoyltetrahydropterin synthase generates the 6-PTP that will be further converted to BH4 by the SR, an important cofactor for the enzymes implicated in the synthesis of dopamine and serotonin. It is an autosomal recessive disorder. There are two clinical presentations regarding their severity: 1) Mild forms with mild intellectual disability and dystonia and 2) severe forms with dystonia-parkinsonism, oculogyric crises and severe intellectual disability.

These patients present low levels of HVA and 5-HIAA and high levels of BP in the CSF.

The treatment of the disorder depends on the severity: severe forms are supplemented with BH4, L-Dopa + carbidopa and 5- hydroxytryptophan whereas the mild forms are not usually treated, although sometimes BH4 supplementation is given (Leuzzi et al. 2010).

Dihydropterine reductase (DHPR) deficiency: The Dihydropterine reductase is the enzyme that regenerates the BH4 cofactor. It is an autosomal recessive disorder that produces microcephaly, hypotonia, tremors, parkinsonism, epilepsy and oculogyric crises. The biochemical profile of the CSF is characterized by low levels of HVA, 5HIAA and BP but high levels of NP. Supplementary treatment with folinic acid is needed: one dose 15-20mg/day. Supplementary treatment with BH4is still controversial, given that there is a tendency in those patients to accumulate BH2, considered neurotoxic and having an inhibitory effect on hydroxylases. Therefore, given that these patients might not have optimal BH4 levels to control plasmatic Phe, a low-Phe diet might be recommended. The supplementation with NT precursors will be needed (Curtis et al. 2012).

Pterin-4a-carbinolamine dehydratase deficiency: Pterin-4a-carbinolamine dehydratase (PCD) deficiency is an extremely rare BH4 disorder. Affected individuals are asymptomatic and show normal psychomotor development, although transient neurologic deficits in infancy have been reported (Thony et al. 1998). Patients may also develop hypomagnesemia and nonautoimmune diabetes mellitus during puberty (Ferre et al. 2014).

1.1.2 Other disorders of monoamine synthesis

Aromatic L-amino acid decarboxylase (AADC) deficiency: The Aromatic L-amino acid decarboxylase is involved in the synthesis of catecholamines (both dopamine and serotonin).
It is an autosomal recessive trait that produces in the great majority of cases a severe encephalopathy, dystonia-parkinsonism and oculogyric crises. Low levels of HVA and 5HIAA as well as high levels of 3-OMD can be found in the CSF analysis. The pharmacological treatment of AADC deficiency is complex and usually has poor to moderate benefits. . The recent guidelines published by Wassengerg (Wassenberg et al. 2017) recommend a combination of inhibitors of monoamine oxidase (IMAO) and dopamine agonists, together with Vitamin B6 and folinic acid (Wassenberg et al, 2017)

Tyrosine hydroxylase deficiency (THD): is described in detail below.

Dopamine beta-hydroxylase (DBH) deficiency: Dopamine beta-hydroxylase is the key enzyme in the synthesis of noradrenaline. It is an autosomal recessive disorder characterized by Orthostatic hypotension and hypoglycaemia. In the CSF, high levels of HVA and low levels of MHPG can be found.

The available treatment consists in L- Threo-DOPS. This compound can be directly converted by AADC into noradrenaline, thereby by-passing the defective en-zyme. Administration of 100 to 500 mg L-Dops orally twice or three times daily in-creases blood pressure and restores plasma NE levels, however plasma epinephrine concentration still remains below a detectable level [65]. The prognosis on therapy is satisfactory to good. (Senard and Rouet 2006).

DnaJ Heat Shock Protein Family (Hsp40) Member C12 (DNACJ12): DNACJ12 Is a chaperone involved in the correct functioning of dopaminergic and serotoninergic synthesis enzymes, and other neurobiological functions still under study.
Mutations in this gene produce hyperphenylalaninemia, dystonia, parkinsonism and in some cases intellectual disability and neurodevelopmental delay.
The biochemical CSF profile analysis revealed low levels of HVA and 5-HIAA. Patients have a good response to dopaminergic (+/- serotoninergic) treatment. (Anikster et al. 2017).

1.2 Disorders of monoamines catabolism:

Monoamine oxidase (MAO) deficiency: Monoamine Oxidase catalyses the catabolism of Dopamine to HVA and Serotonin to produce 5HIAA.
It is rare autosomal recessive disorders that produces psychiatric symptoms, Intellectual disability, aggressiveness, hypotonia and blindness.
Levels of HVA and 5HIAA are low in the CSF and there is an increase of 5-HTP (Ng et al. 2015).
This disease has no effective treatment.

1.3 Disorders of the transport of monoamines:

Vesicular monoamine transporter (VMAT2) deficiency: The vesicular monoamine transporter two facilitates the loading of dopamine and serotonin into synaptic vesicles.
Its defect produces a transportopathy that gives intellectual disability, Dystonia-parkinsonism and dyskinesia. No abnormalities can be found in the CSF of these patients. Treatment with levodopa was associated with worsening, whereas dopamine agonists notably improved the symptoms in patients (Rilstone et al. 2013).

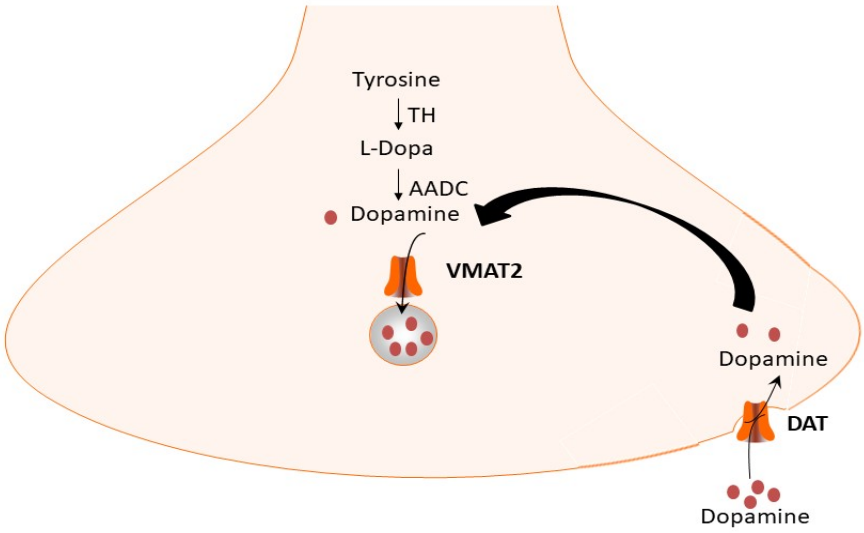
Dopamine transporter (DAT) deficiency: The dopamine transporter is responsible for the presynaptic reuptake of dopamine.
Defects in this transporter (transporthopaty) produce an autosomal recessive disorder with early-onset severe encephalopathy. Patients are described to be hyperkinetic initially, but the gradually

evolve towards a clinical picture of parkinsonism.

The CSF profile is characterized by high levels of HVA. Dopamine agonists are used for the treatment of this disorder although

the clinical response is poor. (Kurian et al. 2011).

Figure 3: Schematic representation of common trasnporthopaties (DAT and VMAT2 deficiencies)



DAT: Dopamine transporter; VMAT2: Vesicular monoamine transporter 2

Table 2: Summary of Classic IEM (Adapted from Tristán-Noguero & García-Cazorla, 2018)

Categories of disease (neurobiological function)	Disease (OMIM)	Biochemical function	Neurological symptoms	Biomarkers (CSF/others)	Bibliography
Synthesis of NT	GTPCH (600225)	Synthesis of monoamines	DRD, Parkinsonism, ID in the AR form	↓HVA, ↓5-HIAA, ↓NP, ↓ BP, ↑PHE (AR)	Opladen et al, 2011
	SR (182125)		DRD, Parkinsonism, OGC, ID	↓HVA, ↓5-HIAA, ↑BP, ↑SP	Bonafé et al, 2001
	PTPS (612719)		D-P, generalized dystonia, OGC. Mild forms: mild ID and dystonia	↓HVA, ↓5-HIAA, ↑NP, ↓BP, ↑PHE	Leuzzi et al, 2010
	DHPR (612676)		Microcephaly, hypotonia, tremors, parkinsonism, epilepsy, OGC	↓HVA, ↓5-HIAA, ↑BP, ↑PHE	Curtis et al, 2012
	TH (191290)		Parkinsonism, D-P, DRD, OGC, severe encephalopathy	↓HVA, ↓MHPG	Willemsen et, 2010
	AADC (107930)		Parkinsonism, D-P, severe encephalopathy, OGC	↓HVA, ↓5-HIAA; ↓MHPG, ↑5-HT, ↑3OMD	Wssenberg et al, 2017
	DBH (609312)		Orthostatic hypotension, hypoglycaemia	↑ HVA, ↓MHPG	Senard & Rouet et al, 2006
Catabolism of NT	IMAO(309850, 309860)	Catabolism of monoamines	Psychiatric symptoms, aggressiveness, ID, blindness	↓HVA, ↓5-HIAA, ↑5-HT	Ng et al, 2015
Transport of NT	DAT (126455)	Transport of monoamines	Most of them severe encephalopathy. D-P, ID	↑HVA	Kurian et al, 2009
	VMAT2 (193001)		intellectual disability, Dystonia-parkinsonism and dyskinesia.	Normal values	Rilstone et al, 2013

2. Tyrosine Hydroxylase deficiency (THD)

2.1 Clinical characteristics

Lüdecke and colleagues describe first this disorder in 1996 (Lüdecke et al. 1996). The authors reported a patient with an early parkinsonism that responded well to the treatment with L-dopa The condition was caused by an hereditary recessive point mutation (L205P) in the tyrosine hydroxylase (TH) gene. From that time, around eighty cases have been reported worldwide.

Although different clinical manifestations and severity degrees have been described, they can be grouped into two main phenotypes:

- Type A: rigid-hypokinetic syndrome (HRS) that can be associated to dystonia and other abnormal movements, with onset in early childhood or later (school age, adolescence).
- Type B: complex encephalopathy that starts at the neonatal period or early childhood; they may have diverse symptoms including HRS, developmental delay, variety of movement disorders and sometimes, epilepsy (although this symptom is rare). Mental retardation, tremors, chorea, oculogyric crises, ptosis, fluctuation of signs, autonomic dysfunction and poor response to L-dopa may be present in both phenotypes, but are more likely in type B. Cognitive and motor prognosis is worse in type B.

2.2 Genetics of Tyrosine Hydroxylase deficiency

The enzyme TH catalyses the rate limiting step in the biosynthesis of catecholamines (converts tyrosine to L-dopa). In patients' CSF, low levels of HVA (homovanillic acid) and MHPG, dopamine catabolites and norepinephrine, respectively, can be found; whereas there is a presence of normal levels of 5-HIAA (5-hydroxyindoleacetic acid, the main metabolite of serotonin) (Willemsen et al, 2010). Diverse missense, promoter mutations and deletions have been described in TH gene according to The Human Gene Mutation Database® (see table 3). It is believed that all these mutations cause a partial loss of TH activity, since it has been suggested that a total loss of TH activity would be incompatible with life. In fact, there are reports of fetal deaths in siblings of affected patients (Møller et al. 2005 and Tristán-Noguero et al. 2016).

Table 3: Summary of genetic mutations described in TH gene according to “The Human Gene Mutation Database”

Gene	Disease	HGMD				
		All mutations	Point mutations		Big rearrangements	
TH	Tyrosine hydroxylase deficiency	34	ms/ns	27 (the more frequents are c.707 T>C p.L236P and c.698 G>A p.R233H; 3 and 10 patients respectively) Willemsen (2010)	gdel	1
			sp	1		
			reg	3 (the most frequent is g-70G>A (5 patients) Verbeek (2007)	cr	1
			sdel	1		

2.3 Disease mechanisms and response to the treatment

Currently, the most important medical problem in this disease is the lack of alternative treatments to dopaminergic drugs, which are not working in severe forms (phenotype B). Although there may be some improvement, the most affected patients have severe cognitive delay and motor disorders that limit their personal autonomy.

In most cases, TH deficiency can be treated with L-dopa in combination with a L-dopa decarboxylase inhibitor. However, the response is variable, ranging from complete remission (more likely in type A) to mild improvement. Therapy should be started with low doses to prevent dyskinesias. The initiating dose should be <0.5 mg/kg per day, with slow titration (over weeks to months) to 3–10 mg/kg per day, according to response. Dyskinesias may be triggered by L-dopa increment, intercurrent febrile illness, tiredness, and overexcitement. It may be successfully treated with amantadine (Pons et al. 2013).

Long lasting and persistent poor DA cerebral innervation seems to be key to the understanding of the pathophysiology of this disease. HVA levels tend to be lower in the most affected patients (Willemsen et al, 2010). Without treatment with L-dopa or other dopaminergic drugs, patients suffer a progressive disease that ends in a severe hypokinetic-rigid syndrome. Patients receiving treatment evolve differently depending on the phenotype.

The standard treatment is L-dopa combined with an inhibitor of AADC decarboxylase (carbidopa). The response is variable, from complete remission (most likely in phenotype A) to a slight improvement (more likely in phenotype B).

"B" patients often have mental retardation and severe motor disabilities including dyskinesias and

Parkinsonian characteristics that have poor or mild responses to L-dopa and other dopamine enhancer drugs. The dyskinesias induced by L- dopa have been related to pre- and post-synaptic changes at the corticostriatal level caused by a chronic and pulsatile stimulation of the receptors.

One of the most intriguing features of this disease is the pathophysiology of the two main phenotypes, and, in particular, the variable response and tolerance to L-dopa, which will affect the overall result. Most THD mutant products are misfolded proteins with reduced stability, solubility and activity (see table 4) . There is evidence that defective gene products, which are usually transcribed at normal levels, can be rescued after translation by pharmacological chaperones.

Table 4: TH functional studies and genotype-phenotype correlations (adapted from Fossbakk et al. 2014)

Table 3. Summary of Features of Mutant TH and Phenotypic Classification of Associated THD (Type A and B)

Mutation	Type THD	Solubility	Residual activity	Thermal stability	Dominant feature	MutPred prediction[a]	$\Delta\Delta G^b$ (kcal mol^{-1})
p.Cys207Tyr[c]	A	Moderately reduced	<20%	Moderately reduced	Activity	0.790, –M*, +P**	2.3:4.0
p.Asp227Gly[c]	A	Not reduced	<0.2%	n.d.	Activity	0.960	6.6:6.7
p.Arg233His[d]	A/B, homozygous		14%	Moderately reduced	Activity	0.980	2.6:2.5
p.Leu236Pro[e]	A/B, homozygous	Reduced	16%[f]		Activity	0.842, –S*	4.5:4.5
			1.5%		Activity		
			0.3% [g]		Activity		
p.Ala241Thr[c]	A/B	Moderately reduced	<5%	n.d.	Activity	0.858	0.56:1.0
p.His246Tyr[c]	A	Not reduced	>50%	Severely reduced	Stability	0.784	–0.35:–0.49
p.Gly247Ser[c]	A	Not reduced	<50%	Severely reduced	Stability	0.797, –C*	4.3:3.7
p.Glu259Gly[c]	A	Sign reduced	n.d.	n.d.	Solubility/stability	0.816	5.3:5.0
p.Thr276Pro[h]	A	–	100%	Sign reduced	Stability	0.845, –S*	–0.19:–0.19
p.Pro301Ala[c]	B, homozygous	Not reduced	<5%	n.d.	Activity	0.892	2.6:2.6
p.Phe309Ser[c]	B, homozygous	Moderately reduced	<0.2%	n.d.	Activity	0.869, –S*, +D**	4.3:4.1
p.Thr314Met[h]	A	–	24%	Severely reduced	Stability	0.956	4.5:4.6
p.Arg319Pro[c]	A	Sign reduced	<0.2%	n.d.	Solubility/stability	0.863, –M*	4.3:4.4
p.Arg328Trp[c]	B	Sign reduced	<0.2%	n.d.	Solubility/stability	0.918, –M*	1.6:1.4
p.Arg337His[h]	A	–	100%	Severely reduced	Stability	0.920	7.7:5.9
p.Cys359Phe[c]	B, homozygous	Moderately reduced	<10%	Severely reduced	Activity, stability	0.720	2.8:4.5
p.Phe375Leu[c]	B	Moderately reduced	<10%	Moderately reduced	Activity	0.962	1.1:1.1
p.Ala376Val[c]	A	Sign reduced	<10%	Moderately reduced	Solubility	0.972	1.9:1.8
p.Leu387Met[c]	A	Moderately reduced	>50%	Severely reduced	Stability	0.881	0.58:0.65
p.Ile394Thr[c]	A	Sign reduced	<0.2%	n.d.	Solubility/stability	0.817, +D*, +P*, +U*	2.8:2.8
p.Thr399Met[c]	B	Moderately reduced	<5%	n.d.	Activity	0.853	0.0:0.1
p.Gln412Lys[e]	A, homozygous	Reduced	18%[f]		Activity	0.808, +M*, +U*	0.33:0.45
			65%		Activity		
p.Gly414Arg[c]	A	Moderately reduced	<10%	n.d	Activity, stability	0.844, –U*	1.8:1.9
p.Arg441Pro[c]	A	Sign reduced	<0.2%	n.d.	Solubility/stability	0.557	3.8:3.8
p.Gln459*[c]	B	Moderately reduced	<0.2%	n.d.	Activity	-	0.87:0.82
p.Ser467Gly[c]	A	Not reduced	<20%	Severely reduced	Thermal stability	0.901, –S*, –D*	1.7:1.6
p.Pro492Leu[c]	B	Sign reduced	<0.2%	n.d.	Solubility/stability	0.884	7.6:1.0
p.Thr494Met[h]	A	Sign reduced	100%		Solubility	0.921	–0.25:–0.83
p.Asp498Gly[c]	A	Sign reduced	<10%	n.d.	Activity, solubility/stability	0.840, –S*	2.7:1.3
p.Leu510Gln[c]	A	Sign reduced	<0.2%	n.d.	Solubility/stability	0.801, +D*	2.3:0.2

2.4 Murine models

Murine models are a reliable source in many cases that allow understand better some mechanism diseases and have been widely used in the last years.

In the case of Tyrosine Hydroxylase several murine models have been generated where they knock

out (KO) or knock in the TH gene.
The first one (Kobayashi et al. 1995) was based on a TH KD and produce and impaired cardiac function and therefore a decrease in perinatal survival. The mice model by Zhou and Palmiter (1995) had a global dopaminergic dysfunction and impaired postnatal survival due to feeding problems.

Later on, Tokuoka and colleagues generated a TH conditional KO,and demonstrated that the axonal TH level and L -DOPA synthesis activity is compensated by striatal dopamine.
Althini et al. 2003 described a TH Ki mouse which homozygous mice were hypokinetic. They show a decrease in TH in situ-hybridization but dopamine and noradrenergic neuronal circuits in the substantia nigra and striatum were preserved Tokuoka et al. 2011.

Finally, two last murine studies were published both the same year: The first one by Rose et al. 2015 harbouring the p.381Q4K TH mutation (c.1141C4A) that caused an A phenotype to the mouse as it was L-dopa responsive. They observed a lower TH expression by immunohistochemistry in striatum and midbrain and low levels of dopamine (DA) metabolites by HPLC studies.

The second one by Korner et al. 2015, described a mice model with the R233H mutation that gave a B phenotype in mice. They observed lower TH expression by Western blot and quantitative PCR studies and low levels of DA metabolites by HPLC.
They also described a mislocalization of the TH in the nigrostriatal pathway, as they found more immunoreactivity of TH in the substantia nigra than in the corpus striatum so they hypothesize about a defective transport of TH across the nigrostriatal pathway, due to a poor enzyme stabilization.

2.5 Cell models

A cell model based on PC12Adh cells (Cells from pheochromocytoma of the rat adrenal medulla) was generated for THD. Three different mutations (p. R202H, p. L205P and p. Q381K) were transfected to these cells where it was possible to observe a reduction on TH expression by means of western blot studies.

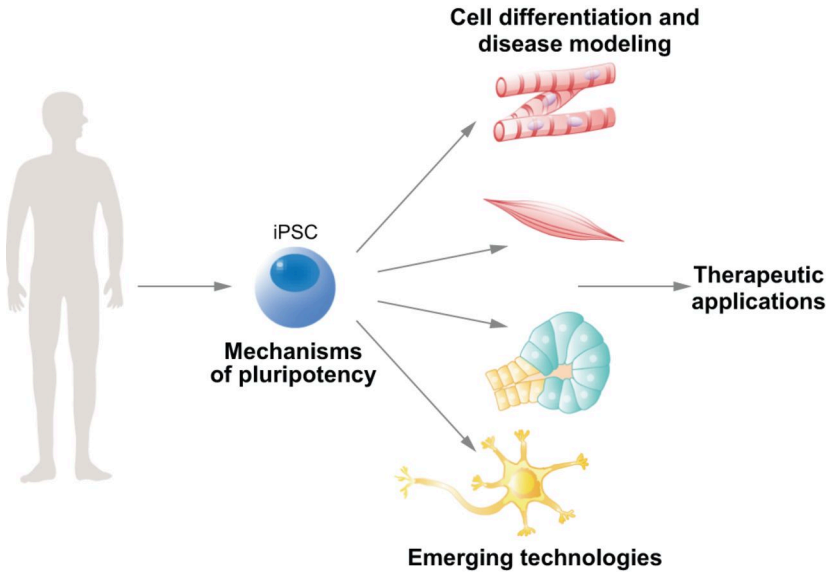
Moreover, this cell model was used to assess the effect of different chaperones (Compound IV 40-100 µM; Compounds 2 and 5, 40 µm) trough TH expression western blot studies and TH activity assays (Hole et al, 2015). The most promising results were found for compound IV and 2 (Hole et al. 2015b).

3. Modelling human brain disorders with Induced Pluripotent Stem Cells

3.1 Induced pluripotent stem cells (iPSCs)

Yamanaka was awarded with the Nobel prize for being able to obtain induced pluripotent stem cells in the laboratory (Takahashi et al. 2007). They successfully reprogrammed human somatic cells (fibroblasts) into a pluripotent state introducing the following factors: Oct3/4, Sox2, Klf4, and c-Myc. This iPSCs are pluripotent cells that allow us to differentiate them in the cell of interest in our disorder (blood, muscle, neurons…).

Figure 4: Schematic representation of the cell type that can derived from iPSCs



The advantages of using iPSCs regarding other diseases models are the following (Hwang et al. 2018):

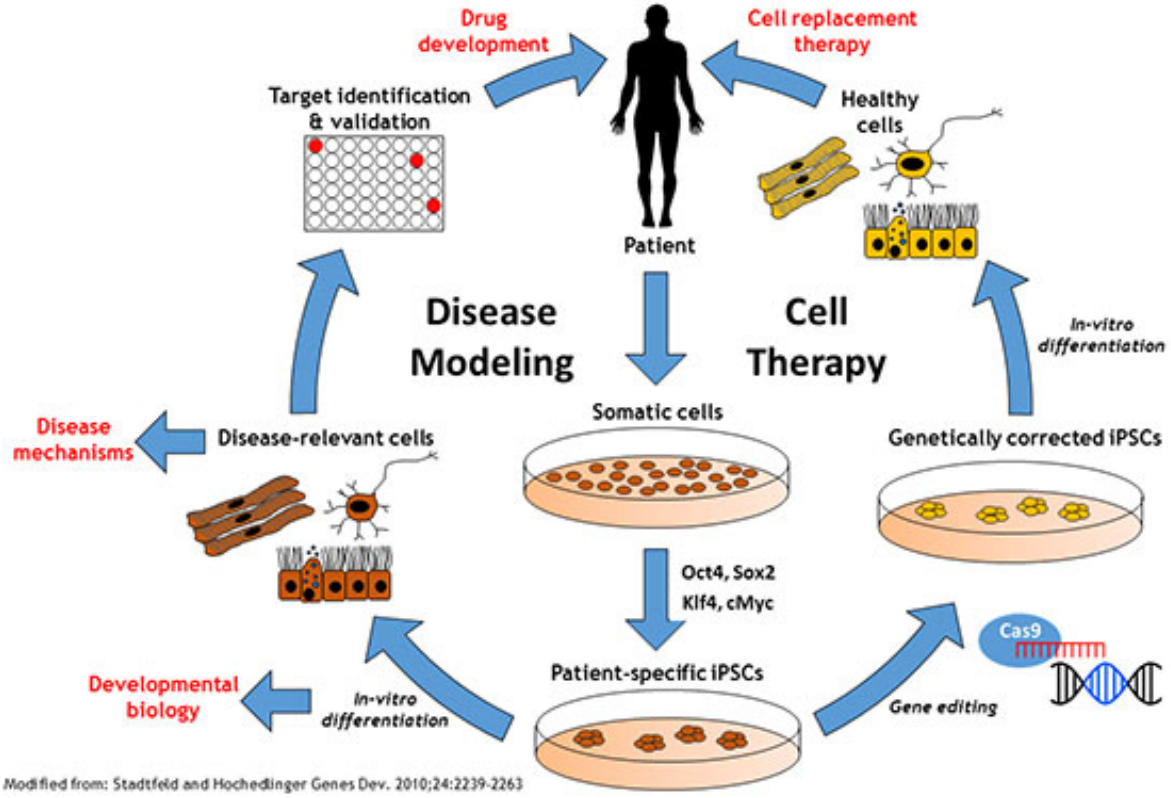
- iPSCs can be maintained in vitro indefinitely harbouring pluripotent state for many generations and with karyotype stability
- IPSCs can be differentiated into all cells in our body allowing to study the cell type of interest
- IPSCs are of human origin and share the genetic background with the patient
- IPSCs can be easily genetically modified to investigate how changes in genome could affect disease

- iPSCs also offer a better alternative to animal models to test drugs and toxicity as they mimic better the human nervous system

iPSCs may have been used for different purposes: study disease mechanisms, drug development or cell replacement therapy among others.

It is also possible to genetically edit this iPSCs with different editing technologies such as talents or Crispr-Cas9 to introduce one specific mutation in a control line or correct the mutation of a disease cell line to obtain what it is usually called an isogenic control.

Figure 5: Schematic representation of the different possible uses of iPSCs:



iPSCs technology has been used for modelling different disorders since were first described. Initially genetic but later on also metabolic diseases (Canals et al. 2015) or neurodegenerative disorders

In the case of neuronal diseases, on one hand mouse model don't usually explain all the disorders' phenotypes and in the other hand it is complicated to gather patient samples.

Moreover, these samples sometimes don't truly reflect what happens really in the brain or is at later stages in the case of neurodegenerative disorders (neurodegeneration is complete and lead to death) (Torrent et al. 2015):

In those cases, is especially interesting to develop a model based on ips. There are different

methods to fulfill this and obtain Dopaminergic neurons (Sanchez-Danes et al. 2013):

- Coculture with feeder cells; for example PA6 stromal murine cells or primary astrocytes
- EB's formation
- Direct lineage conversion with small molecules or recombinant proteins

3.2 Use of iPSCs for modelling childhood neurological disorders

There are several studies where iPSCs has been used for modelling a huge variety of childhood neurological disorders. From neurodevelopmental disorders to movement or metabolic disorders (Barral and Kurian 2016) see table 5.

Table 5: Using iPSCs in childhood-onset neurodevelopmental and neurological disorders (From Barral and Kurian 2016)

Disease	Gene(s)	Differentiated cell type	Molecular characterization	Compound screening	Gene/RNA therapy
Neurodevelopmental disorders					
Rett syndrome	MEPC2 CDKL5	Neural progenitor cells Neurons (glutamatergic) Astrocytes	Muotri et al. (2010), Amenduni et al. (2011), Ananiev et al. (2011), Cheung et al. (2011), Kim et al. (2011), Farra et al. (2012), Larimore et al. (2013), Williams et al. (2014), Andoh-Noda et al. (2015), Djuric et al. (2015), Fernandes et al. (2015), Livide et al. (2015), Tang et al. (2016) and Zhang et al. (2016)	Marchetto et al. (2010)	
Fragile X syndrome	FMR1	Neural precursor cells Neurons (forebrain) Glial cells	Urbach et al. (2010), Sheridan et al. (2011), Doers et al. (2014) and Halevy et al. (2015)	Kaufmann et al. (2015) and Kumari et al. (2015)	Park et al. (2015)
Microcephaly	ERCC6 CDK5RAP2	Neurons Cerebral organoids	Lancaster et al. (2013) and Vessoni et al. (2016)		
Angelman/Prader-Willi syndromes	UBE3A	Neurons Astrocytes	Chamberlain et al. (2010)		
Timothy syndrome	CACNA1C	Neural progenitor cells Neurons	Krey et al. (2013) and Tian et al. (2014)	Paşca et al. (2011)	
Phelan-McDermid syndrome	Chromosome 22q13 deletion	Neurons (forebrain)		Shcheglovitov et al. (2013)	
Epilepsy					
Dravet syndrome	SCN1A	Neurons (dopaminergic, GABAergic) Forebrain interneurons Glutamatergic neurons	Higurashi et al. (2013), Jiao et al. (2013), Liu et al. (2013, 2016) and Maeda et al. (2016)	Jiao et al. (2013)	

Disease	Gene(s)	Differentiated cell type	Molecular characterization	Compound screening	Gene/RNA therapy
		Glial cells			
Early infantile epileptic encephalopathy	STXBP1	Neurons (glutamatergic, GABAergic)	Yamashita et al. (2016)		
Movement disorders					
Hereditary spastic paraplegia	SPG11 ATL1 SPAST	Cortical neural progenitor cells Neurons (forebrain glutamatergic)	Denton et al. (2014); Havlicek et al. (2014) and Mishra et al. (2016)	Zhu et al. (2014)	
Ataxia telangiectasia	ATM	Neural progenitor cells Neurons (GABAergic)	Nayler et al. (2012) and Carlessi et al. (2014)	Lee et al. (2013)	
Friedrich's ataxia	FXN	Neural progenitor cells Neural crest cells Neurons (peripheral sensory) Glial cells	Liu et al. (2011), Eigentler et al. (2013), Hick et al. (2013) and Bird et al. (2014)	Shan et al. (2014), Soragni et al. (2014) and Igoillo-Esteve et al. (2015)	Li et al. (2015)
Huntington's disease	HTT	Striatal neural precursor cells Neurons (GABAergic striatal) Astrocytes	Camnasio et al. (2012), Chae et al. (2012), HD iPSC Consortium (2012), Jeon et al. (2012), Juopperi et al. (2012), Mattis et al. (2015) and Szlachcic et al. (2015)	Guo et al. (2013), Hsiao et al. (2014) and Lu et al. (2014)	An et al. (2012) and Cheng et al. (2013)
Metabolic disorders					
Lesch-Nyhan syndrome	HPRT	Neurons	Mastrangelo et al. (2012) and Mekhoubad et al. (2012)		
Niemann-Pick type C disease	NPC1	Neurons Astrocytes	Trilck et al. (2013, 2016)	Efthymiou et al. (2015)	
Neuronal ceroid lipofuscinosis disease	TPP1 CLN3	Neurons	Lojewski et al. (2014)		
Gaucher's disease	GBA1	Neurons (dopaminergic)	Awad et al. (2015) and Sun et al. (2015)	Tiscornia et al. (2013)	
Metachromatic leukodystrophy	ARSA	Neural stem cells Astroglial progenitor cells	Doerr et al. (2015)		
X-linked Adrenoleukodystrophy	ABCD1	Neurons Astrocytes Oligodendrocytes	Jang et al. (2011) and Baarine et al. (2015)		
Neuromuscular disorders					
Spinal muscular atrophy	SMN1	Neurons (motor neurons, forebrain neurons, sensory neurons) Astrocytes	Ebert et al. (2009), Chang et al. (2011), McGivern et al. (2013), Schwab and Ebert (2014), Boza-Morán et al. (2015), Demestre et al. (2015), Liu et al. (2015), Ng et al. (2015), Fuller et al. (2016), Heesen et al. (2016) and Patitucci and Ebert (2016)	Sareen et al. (2012), Ohuchi et al. (2016) and Xu et al. (2016)	Corti et al. (2012), Nizzardo et al. (2015) and Yoshida et al. (2015)

3.3 Use of iPSCs for modelling metabolic disorders

iPSCs technology has been also widely used for the modelling of different metabolic disorders; most of them related to hepatic metabolism diseases such as glycogen storage diseases, Wilson's disease, Niemman-Pick Type C or Alper's disease with promising results in recapitulating the associated phenotype and in drug discovery (Pournasr and Duncan 2017) (see table 6).

Table 6: Inherited metabolic liver diseases modelled with iPSCs (adapted from Pournasr and Duncan 2017)

Liver Disease	Pathophysiology	Defective Protein	iPSCs-Based Disease modelling	Ref
Alpha-1-Antitrypsin Deficiency (A1ATD)	Increasing circulating level of A1ATD, Neonatal cholestasis, Liver failure	Single gene defect, mutation in A1ATD	Patient derived iPSCs differentiated to hepatocytes	Yusa et al, 2011
Glycogen Storage Diseases (GSD1A and GSD1B)	Hypoglycemia, Neurological defect	G6PC, Glucose-6-Phosphatase Catalytic Subunit (GSD1B) SLC37A4, Glucose-6-Phosphate Transport Protein 1 (GSD1B)	Patient derived iPSCs differentiated to hepatocytes	Rashid et al, 2010
Tangier Disease, Familial alpha-lipoprotein deficiency	Severely reduced high-density lipoprotein (HDL)	ABCA1, ATP-Binding Cassette Transporter A1	Patient Derived iPSCs differentiated to hepatocytes	Bi et al, 2017
Tyrosinemia Type I(TTI)	Accumulation of toxic compound, Hepatocyte and renal tubular cells death	FAH, Fumarylacetoacetate Hydrolase	Patient derived iPSCs differentiated to hepatocytes	Rashid et al, 2010
Familial Hypercholesterolemia (FH)	High level of circulating LDL, Coronary artery disease and Myocardial infection	LDLR, Low Density Lipoprotein Receptor	Patient derived iPSCs differentiated to hepatocytes	Cayo et al, 2012
Wilson's Disease (WD)	Accumulation of too much copper in liver, brain and some other vital organs.	ATP7B, Copper-Transporting ATPase 2	Patient derived iPSCs differentiated to hepatocytes	Zhang et al, 2011
Alper's Disease	Neurodegenerative disease with occasional liver failure	POLG	Patient derived iPSCs differentiated to hepatocytes	Li et al, 2015
Crigler-Najjar Syndrome	Hyperbilirubinemia	UGT1A1, UDP-Glucuronosyltransferase 1-A	Patient derived iPSCs differentiated to hepatocytes	Ghodsizadeh et al, 2010
Niemann-Pick Type C	Lysosomal storage disease	Progressive neurological disease, occasional hepatomegaly	TALEN mutated iPSCs	Maetzel et al, 2014

In the case of inborn error disorders of neurotransmitters there is only one paper (Ishikawa et al. 2016) where they generated iPSCs from two patients with BH4 disorders. They derived iPSCs from one a patient with PTPS deficiency and another with DHPR deficiency and they compare the results with two isogenic controls obtained with CRISPS/CAS9 technology.

In the case of PTPS deficiency derived DAn they show a reduced % of TH-positive neurons, less TH

stained area, lower TH expression when assessed by Western blot, decreased levels of extracellular DA and less BH4 but higher neopterin levels when compared to its isogenic control.

In the case of DHPR deficiency derived DAn they also showed a reduction of TH protein expression, low levels of extracellular dopamine and higher levels of BH2 and biopterin in comparison with the isogenic ones.

PTPS iPSCs were used to test different therapeutic approaches such as BH4, sepiapterin and L-Dopa.
In the case of sepiapterin and BH4 treatments, it was possible to observe an increase in the TH stained area, increased TH protein expression and higher levels of extracellular DA. The treatment of L-dopa alone also increased TH expression and extracellular DA levels. A potentiation of sepiapterin effect on the levels of extracellular DA was observed when administered together with L-Dopa (additive effect).

OBJECTIVES

1. To evaluate the expression of different neuronal markers and proteins of the principal neurotransmitter pathways (dopaminergic, glutamatergic and GABAergic) in a 16 weeks THD human brain and compare the results to an aged-matched control brain.
2. To describe the expression of protein biomarkers (proteomics) in the cerebrospinal fluid (CSF) from patients with different neurotransmitter defects:
    i. Assess differences between disorders
    ii. Characterize the degree of severity of patients
    iii. Characterize the patients' response to the existent treatments.
3. Generate a new THD cell model based on Induced Pluripotent Stem Cells (iPSCs) of two THD patients (one with A phenotype and another with B phenotype) to better understand the physiopathology of this disorder and test different therapeutic approaches in vitro:
    i. Generate iPSCs from the two patients and two controls and differentiate them to DA neurons
    ii. Describe the disorder associated phenotype in both patients when compared to the controls and check also the differences among the two patients' phenotypes
    iii. Correct the THD A patient mutation with CRISPR/CAS9 technology so as to obtain an isogenic control and confirm the robustness of genetic and mutation related changes.
    iv. Test different therapeutic approaches in vitro (L-dopa + carbidopa and new treatments such as chaperones) in our THD iPSCs model.

MATERIALS AND METHODS

1. Tyrosine Hydroxylase deficiency (THD) postmortem brain

1.1 Samples preparation

The brain tissue of a 16-week-old miscarried fetus was dissected in three different cortical areas (parietal, occipital and temporal), mesencephalon, pons and cerebellum that were immediately frozen at −80 °C together with the suprarenal gland (SG). TH gene study revealed the same p. R328W/p. T399M mutations carried by her 17-year-old sister (Møller et al. 2005). The brain of an age-matched control fetus obtained

after a spontaneous miscarriage was dissected and stored following the same procedure.

1.2 Proteins extraction

Proteins were extracted with lysis buffer using the bullet blender to homogenize the sample (Zirconium oxide beads 0.5 mm diameter). Protein concentration was determined by the Bradford method and protein extracts were stored at - 80 °C until the moment of analysis.

1.3 Western Blot studies

Samples were prepared with Laemmli Sample Buffer (10 % DTT). 12 or 24 µg of protein extract were separated on 8 or 12 % SDS-PAGE gels (depending on the protein of study) and transferred to a nitrocellulose membrane. Membranes were blocked with 5 % milk (prepared with PBS Tween) for a duration of

15 min and were incubated with the corresponding primary antibody at 4 °C (see table 7).

A secondary peroxidase-conjugated antibody was used during 1 h at room temperature.

The blot was incubated with ECL and exposed to X-ray film. Optical density value of each band (Quantity One v 4.3.1 software) was corrected by the β-actin (Sigma-Aldrich) value. After that, the value of each protein was measured relative to the control (this being 1).

Table 7: Information of the antibodies used in this study:

Antibody	Species	Reference	Dilution	Weight (KDa)
Tyrosine Hydroxylase (TH)	Mouse	MAB218 (Merck Millipore)	1:1000	60
Vesicular monoamine transporter 1 (VMAT1)	Rabbit	AB1597P (Merck Millipore)	1:1000	55
Vesicular monoamine transporter 2	Rabbit	sc-15314 (Santa Cruz)	1:500	53

(VMAT2)				
Aromatic L-amino acid decarboxylase (AADC)	Rabbit	Ab3905 (Abcam)	1:1000	45
Dopaminergic receptor 1 (D1DR)	Rabbit	Ab81296 (Abcam)	1:1000	53
Dopaminergic receptor 2 (D2DR)	Rabbit	AB5084P (Merck Millipore)	1:1000	55
Calbindin	Rabbit	AB1778 (Merck Millipore)	1:5000	28
Vesicular transporter of GABA (GABA VT)	Rabbit	AB5062P (Merck Millipore)	1:1000	57
N-methyl-D-Aspartate receptor 1 (NMDAR1)	Mouse	ab134308 (Abcam)	1:1000	105
Postsynaptic density 95 protein (PSD95)	Rabbit	ab18258 (Abcam)	1:1000	90
Phosphorylated neurofilament H (NF-H)	Mouse	NE1022 (Merck Millipore)	1:1000	110
Microtubule associated protein 2 (MAP2)	Rabbit	8707P (Cell signalling)	1:1000	75
Neuron specific enolase (NSE)	Mouse	MAB324 (Merck Millipore)	1:1000	47

2. Proteomics study

2.1 Samples included in the study:

92 cerebrospinal fluid samples (CSF) were recruited from different countries in a joint collaboration with the international consortium: International Working Group on Neurotransmitter Related Disorders (iNTD). iNTD is an international working group for the study of neurotransmitter related disorders composed by 42 centres in 26 countries worldwide: http://intd-online.org (Opladen et al. 2016),

This population includes patients with diverse neurotransmitter defects, in detail: 29 TH, 17 AADC, 9 GTPCH, 8 DHPR, 22 PTPS, 4 SR and one DAT and 2 unknowns with ages from 0 to 30 years (average age of 6 years). Of these patients, 39 were males and 51 females.

The following variables were collected and assessed in these patients: Age, sex, disease, severity, mutation, biochemical values and treatment.

2.2 Proteomics study

The proteomics analysis was performed using the following workflow: First a complete randomization of the samples was done so as to not influence the results of the study. Second, the CSF samples were precipitated and digested with trypsin to obtain the separate peptides. Finally the data was acquired by an independent manner through mass spectrometry using the Orbitrap Fusion Lumos equipment (DIA Umpire v2.1).

2.3 ELISA validation

We selected 6 proteins with differential abundance in the population of study when it was divided in different groups regarding the categories of study. We selected OMGP and APOH for THD; NEUM and APOD for AADC patients and NFASC and COL6A3 for the group of BH4 disorders. Those proteins were validated by a second protein detection technique such as an enzyme linked immunosorbent assay (ELISA).

Briefly, 100 ul of the CSF sample (not diluted or diluted 1/5) was charged in the ELISA plate precoated with a primary antibody against the protein of interest. Followed by an incubation with a secondary antibody conjugated with HRP obtaining a colorimetric reaction when the substrate was added. This colour reaction was quantified by a plate reader at 450 nm which gives us an optic density value that correlates with the protein concentration in the sample (see table 8).

Table 8: ELISA kits used in this study:

Protein	Reference	Sample dilution
Oligodendrocyte-myelin glycoprotein (OMGP)	126ELH-OMGP (RayBiotech)	Not diluted
Apolipoprotein H (APOH)	271EH0531 (Finetest)	1/5
Neuromodulin (NEUM)	271EH1497 (Finetest)	Not diluted
Apolipoprotein D (APOD)	271EH2135 (Finetest)	1/5
Neurofascin (NFASC)	CSB-EL015743HU (Causabio)	Not diluted
Collagen Type VI alfa 3 (CO6A3)	271EH2871 (Finetest)	Not diluted

2.4 Statistical analysis

An ANOVA test with the R package was used to test the correlation between the abundance of certain proteins with the different variants of the study. The data was represented using box plots graphs.

We used the Prism software (Mac OS X) to perform a second test (ANOVA, t-test or Mann-Whitney test) to validate the results of the ELISA kits.

3. Cell model based on iPSCs

3.1 General cell culture protocols

3.1.1 IPSCs generation and cell culture

After informed consent was obtained, fibroblasts from 2 THD patients and 2 healthy controls were obtained by a punch biopsy. Those fibroblasts were reprogrammed to iPSCs using a non-integrating episomal vector expressing Oct4, Sox2, Nanog, Klf4, c-myc and Lin28 to generate 2-4 independent iPSCs lines per individual, that were thoroughly characterized (AP staining, karyotype stabilization, mutation analysis, positive staining for pluripotent markers and ability to generate the 3 germ layers).

The generation and use of human iPSCs in this work were approved by the Spanish competent authorities (Commission on Guarantees concerning the Donation and Use of Human Tissues and Cells of the Carlos III National Institute of Health

The iPSCs were maintained in mTeSR1 medium and Matrigel coating and passage once a week with EDTA.

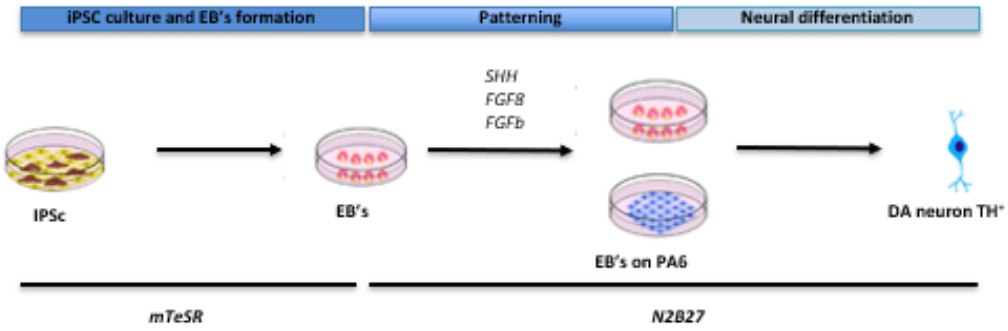
3.1.2 Generation of embryoid bodies (EBs):

When the iPSCs cell cultures were confluent, cells were detached with EDTA and seed on 96 with V bottom to force aggregation in shape of embryoid bodies (EBs). Those EBs were taken out from the plate and maintained with mTeSR1 medium for one to two days until they were well-formed. Then were induced for 10 days with dopaminergic patterning factors (FGF2, SHH & FGF8) in N2B27 media (Neurobasal, DMEMF12, N2, B27, P/S, glutamax) changing the media and factors every two days.

3.1.3 Dopaminergic differentiation:

We wanted to obtain Dopaminergic neurons as they are our cell of interest in this disorder. So, After the 10 days induction of the EBs, they were seed on top of PA6 (stromal murine cells that act as feeders) and maintained for 3 weeks in N2B27 media changing the media 3 times per week. Once we obtained these neurons they were used for the different assays.

Figure 6: Schematic representation of the DA differentiation protocol



3.2 Western Blot (WB) studies:

Cells were harvested with cold PBS and after their centrifugation (5' 2000 rpm), the pellet was treated with RIPA buffer supplemented with protease inhibitor (10X) to extract proteins. After that the pellet was sonicated (amplitude 100 during 10 seconds) and let in a rotator during 1 h approximately at 4 ºC to homogenize proteins.
Protein concentration was determined by the Bradford method and protein extracts were stored at -80º until the moment of analysis.

Samples were prepared with Laemmli Sample buffer (10% DTT). 15 µg of protein extract were separated on 8% SDS-PAGE gels and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk (prepared with TBS-tween) during 1h at room temperature and incubated with the TH primary antibody (Santa Cruz, sc-14007) ON at 4ºC. A secondary peroxidase-conjugated antibody (anti-rabbit) was used during 1hour at room temperature. The blot was incubated with ECL and revealed with the Chemidoc (BioRad). The expression levels were quantified with Image J after substracting the background. The optical density value of each band was corrected by the value of β-actin (Proteintech, 66009-1-Ig).

3.3 High Performance Liquid Chromatography (HPLC) studies:

The supernatant or cell media of 3 weeks dopaminergic differentiation on top of PA6 was harvested and kept directly at -80 ºC until the moment of analysis.

Before analysis them, the medium samples were previously deproteinised with an homogenization medium (50 ul), centrifuged at 3750 rpm during 30' at 4ºC and the supernatant was filtered (Millex-HV 0,45um Millipore filters) for a posterior HPLC injection.

Homogenization medium recipe:

- 100 mL miliQ H_2O
- 100 mg of sodium metabisulphite (Sigma S-1516)
- 10 mg EDTA-Na (Sigma E5134)
- 100 mg cysteine (Sigma C-4022)
- Add 3,5 mL de HClO4 concentrated (Scharlau, 70%)

The concentration of DA and 3,4-Dihydroxyphenylacetic acid (DOPAC) in supernatant (SN) samples was determined by an HPLC system consistent of a Waters 717plus autosampler (Waters Cromatografia, Cerdanyola, Spain), a Waters 515 pump, a 2,6-um particle size C18 column (7,5x0,46 cm, Kinetex, Phenomenex, Madrid, Spain) and a Waters 2465 amperometric detector set at an oxidation potential of 0.75 V. The mobile phase consisted of 0.15M NaH2PO4, 0.9mM 1-octane sulfonic acid, 0.5mM EDTA (pH 2.8, adjusted with phosphoric acid), and 10% methanol and was pumped at 0.8 ml/min. The total sample analysis time was of 20min and the DA and DOPAC retention times were 2.17min and 2.35min respectively. The detection limit for DA was of 2fmol (injection volume 20 µl).

Corresponding dopamine metabolite content was normalized to protein concentration determined previously by Bradford method detection.

3.4 Elisa to measure intracellular Dopamine:

Cells were harvested with cold PBS and centrifuged at 2000 rpm during 5 minutes. The obtained pellet was then diluted in 135 ul of a Na metabisulfite and EDTA solution. Later on, the pellet was sonicated with an amplitude of 100 in a 1-minute cycle (sonication during 10 seconds and left in ice during 10 seconds; the process was repeated three times).

115 ul of the 135 ul were kept for ELISA studies and the left volume was used to determine protein concentration with the Bradford method. Those samples were kept at -80ºC until the

moment of analysis.

The intracellular dopamine was measured using an ELISA kit for Dopamine (BA E-5300, LDN) by duplicates. This determination is realised in two parts, first DA is extracted, it is acetylated and activated and then is enzymatically converted and detected with a competitive ELISA assay.

3.5 Molecular studies

3.5.1 RNA extraction

The isolation of total mRNA was performed using the RNAeasy Mini kit from Qiagen after passing the sample through a 20 G gauge for their correct homogenization. The RNA concentration and purity were assessed using the nanodrop.

3.5.2 Retrotranscriptase PCR (RT-PCR):

500ng of total mRNA were used to synthesize cDNA with the SuperScript III Reverse Transcriptase Synthesis Kit (thermofisher).

Mix for de cDNA synthesis:

MIX 1:
1 µl OligodT primers
1 µl dNTPs 10mM mix
RNA (µl depending on its concentration)
H2O (up to 10 µl)

MIX 2:
2 µl 10 x RT buffer
4 µl 25mM MgCl2
2 µl 0,1M DTT
1 µl RNaseOut 1
1 µl Superscript III RT

Programme used:

1. Mix 1: 65ºC 5’
2. Put on ice during 1 minute
3. Add 10 µl of the mix 2
4. 50ºC 50’
5. 85ºC 5’

The RT-PCR product was diluted with RNAse free water to obtain a final concentration of 2 ng/ µl.

3.5.3 Quantitative PCR (qPCR):

Quantitative RT-PCR analysis was done in duplicate using 2ng/ul cDNA with PowerUp SYBR Green Master Mix (thermofisher) in an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems). All results were normalized to β-actin.
4 µl of cDNA and 6 µl of the qPCR mix were charged:

Mix qPCR: 5 µl PowerUp SYBR Green
0,8 µl Mix primers (F+R)
0,2 µl H_2O

Programme used:

90 ºC 2'
95 ºC 2'
95 ºC 15''
60 ºC 1'

In the case of using new primers, a disassociation curve was performed to test the quality of these primers using the following conditions:

95 ºC 15'' (ramp: 1,6ºC/second)
60 ºC 1' (ramp: 1,6ºC/second)
95 ºC 15'' (ramp: 0,15ºC/second)

Table 9: Primers used for the gene's expression analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Tyrosine Hydroxylase (TH)	TGTAAGCAGAACGGGGAGGT	AGCTTGTCCTTGGCGTCACT
Vesicular monoamine transporter 2 (VMAT2)	ATGAGTTTGTGGGGAAGACG	TGTTTGCAAAGCAGATGGAG
Aromatic L-amino acid decarboxylase (AADC)	GAACAGACTTAACGGGAGCCTTT	AATGCCGGTAGTCAGTGATAAGC

Dopaminergic receptor 1 (D1DR)	CTTAGGATGCTACAGACTTTGCCCTG	CATGTGGGATCAGGTAAACCAGATTG
Dopaminergic receptor 2 (D2DR)	TCTTCGGACTCAATAACGCAGACC	GATGTAGACCAGCAGGGTGACAAT
Dopamine transporter (DAT)	ATCCTGCAATGGGAGAGACACGAA	ATTACAGCAACACAAGACACGGCG
N-methyl-D-Aspartate receptor 1 (NMDAR1)	GTCCACCAGACTGAAGATTGTGAC	CTCCTCCTTGCATGTCCCA
N-methyl-D-Aspartate receptor 2A (NMDAR2A)	GGGCTGGGACATGCAGAAT	CGTCTTTGGAACAGTAGAGCAA
N-methyl-D-Aspartate receptor 2B (NMDAR2B)	GGTCTTCTCCATCAGCAGAGG	TGTTGTTCATGGTTGCGGT
Neuron specific enolase (NSE)	GGCTACACGGAAAAGATCGTTATT	GAAGGATCAGTGGGAGACTTGAA
Beta actin	AGGCCAACCGCGAGAAG	ACAGCCTGGATAGCAACGTACA

3.6 Genetic edition with CRISPR/CAS9 system

Three CRISPR/Cas9 gRNAs targeting the exon 6 of human TH gene were R233H mutation locates were designed (sequences below). An ssODN with the mutation corrected, the PAM sequence inactivated and a new enzyme restriction site for HindIII was also designed.

gRNA:

TH_ex6_gRNA1_F: CACCGACCAGGTGTACCGCCAGC**A**C

TH_ex6_gRNA1_R: AAACG**T**GCTGGCGGTACACCTGGTC

TH_ex6_gRNA2_F: CACCGCAATCAGCTTCCTG**T**GCTGG

TH_ex6_gRNA2_R: AAACCCAG**A**GCAGGAAGCTGATTGC

TH_ex6_gRNA3_F: CACCGCAGCAATCAGCTTCCTG**T**GC

TH_ex6_gRNA3_R: AAACGC**A**CAGGAAGCTGATTGCTGC

ssODN_repair_ex6_Bottom_corrected:

GGGTCCCGAGCGCAGGGGCCCCTCACTGCCTGTACTGGAAGGCGATCTCAGCAATAAGCTTTCTGCGCTGGCG
GTACACCTGGTCCGAGAAGCCCTGAGGG

3.6.1 Endonuclease T7 assay

We used the endonuclease t7 assay to assess with gRNA had the highest cleavage efficiency following the formula:

$$\%\text{gene modification} = 100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$$

The chosen gRNA was kindly provided by Synthego.

3.6.2 Nucleofection:

THDA1#17 iPSCs were nucleofected with the 4D AMAXA nucleofector with the chosen gRNA together with the CRISPR and CAS9 proteins (Thermofisher) that formed a RNP complex. Briefly, 200.000 cells were nucleofected with the following mix:

16,4 µl solution
3,6 µl supplement (nucleofection buffer)
6 µl gRNA (30 µM)
→ Incubate 10' and add 1,8 µl de ssODN (50 µM)

3.6.3 Selection of clones

After the nucleofection, we left the cells to recover. We then performed a first screaning to check if those cells had been edited. Once confirmed, they were cultured as single cells to obtain monocolonal colonies. They were seeded on a 96 well plate to be able to peak isolated clones and check again if it had been edited and their clonality (see procedure below).

3.6.4 DNA extraction, PCR, digestion and sequencing

After confirming the edition of the pooled of cells, a DNA extraction was performed of these single clones to perform a PCR of the exon 6 and the following HindIII digestion to check which clones had been edited. The ones that were digested with HindIII were afterward sanger

sequenced to check if the mutation had been corrected (protocols below).

DNA extraction

1. Centrifuge resuspended cells at 8000 rpm during 5' and discard the supernatant
2. Add 48 µl of lysis buffer → Put at 55 ºC during 20' in the termobloc (agitation)
3. Add 10 µl of NaAc and 39 µl of isopropanol → vortex
4. Centrifuge at 4ºC at 14000 rpm during 10' and discard the supernatant
5. Add 100 µl of 70% ethanol and centrifuge at 4ºC at 15000 rpm during 15'
6. Aspirate the liquid with the vacuum and let dry at room temperature during 10'
7. Resuspend with TE buffer (15-25 µl) a 37ºC
8. Measure concentration and purity with the Nanodrop

PCR:

Mix PCR:

2,5 µl gDNA
2,5 µl Buffer
1 µl $MgCl_2$
0,5 µl Primer F (exon 6_T7 F)
0,5 µl Primer R (exon 6_T7 R)
0,25 µl dNTPs

PCR programme:

95 ºC 2'
95 ºC 2''
59 ºC 30''
72 ºC 40''
72 ºC 4'
16 ºC

HindIII digestion:

0,3 µl HindIII restriction enzyme
0,5 µl Buffer green

4, 2 µl H_2O

➔ Put 1h at 37 ºC

DNA Sequencing:

The PCR of positive clones (digested with HindIII) was repeated with a final volume of 25 µl. After its purification with columns (protocol below), we sequenced 4 µl of cDNA + 0,4 µl primer T7 Forward or Reverse.

The sequences were sent to the PCBs Genomic services where they performed the capillary electrophoresis with the automatic sequencer ABI PRISM 3730 (Applied Biosystems). The obtained chromatograms were analysed with the software SnapGene Viewer.

PCR purification (QIAquick PCR purification kit):

1. Add 5 volumes of buffer PB to 1 volume of the PCR
2. Place a QIAquick column in a collection tube and apply the sample to the column and centrifuge at 13000 rpm for 30-60s
3. To wash, add 750 µl of buffer PE to the QIAquick column and centrifuge at 13000 rpm for 30-60s
4. Centrifuge the QIAquick column in a new collection tube for 1 min to remove residual wash buffer
5. Place each QIAquick column in a clean 1,5 ml microcentrifuge tube
6. To elute DNA, add 20-50 µl of buffer EB (previously heated at 55 ºC) leave it 5 minutes and centrifuge for 1 minute
7. Measure the concentration and quality in the Nanodrop

3.7 Treatments

3.7.1 Chaperone

The chaperone (compound 2) was provided by a collaborator (Aurora Martínez) and was resuspended in 100% DMSO and sonicated for their total resuspension to obtain a 16 mM stock concentration.

We performed different trials using different concentrations dose of this peptide diluted in DMSO and the corresponding mock (DMSO should be kept at 1%). The cells were treated during the dopaminergic patterning in form of EBs (first 10 days) and maintained during the 3 weeks of differentiation, adding the chaperone with the same media changes.

3.7.2 L-Dopa + carbidopa

L-dopa (3,4-Dihydroxy-L-phenylalanine, D9628) and carbidopa (PHR1655) were both bought from Sigma Aldrich.

Both components were diluted in cell culture water to obtain a 1mM concentration stock, L-Dopa been kept from light.

DAn cell cultures were treated with both components and the corresponding mock (cell culture water) during the last 10 days of differentiation when the neurons are already formed and are reaching maturity.

3.8 Immunocytochemistry (ICC) studies:

Samples were fixed with 4% PFA for 20 minutes and washed three times for 15 minutes with PBS. Samples were blocked with TBS ++ (0,3% TRITON X-100 + 3% donkey serum) for blocking.

The primary antibody was incubated ON during 48h at 4ºC (see table 10).

Samples were then washed with TBS + (0,1% triton) three times during 15 minutes, and blocked again during 1 hour previous to incubate 2h at RT with the secondary fluorescent (see table 11) and DAPI to stain the nucleus.

Finally, the coverslips were mounted with PVA: DABCO and stored at 4ºC until imaged. Samples were imaged Carl Zeiss LSM880 (Zeiss) and analysed using Image J.

Table 10: Primary antibodies used for the immunocytochemistry studies:

Antibody	Species	Reference	Dilution
Tyrosine Hydroxylase (TH)	Rabbit	sc-14007 (Santa Cruz)	1/250
Tubulin ß 3 (Tuj1)	Mouse	MMS-435P (Covance)	1/500

Table 11: Secondary antibodies used for the immunocytochemistry studies:

Antibody	Species	Reference	Dilution
Cy3	Anti-rabbit igG	715-545-152 (Jackson)	1/200
Alexa Fluor 488	Anti-mouse igG	715-545-150 (Jackson)	1/200

3.9 Image analysis

3.9.1 Cells counting

We counted the nuclei and the positive cells for the different markers in each experiment to obtain the number of cells and neurons in the culture and therefore calculate the ratios TH/DAPI TUJ1/DAPI and TH/Tuj1 with the cell counter plugin of the Image J programme.

3.9.2 Fiber density

A mask was generated to see how much area of the image was occupied by the TH + fibers and neurites discarding the area occupied by the nuclei. The area was corrected by the number of neurons present in each image.

3.9.3 TH transport

The mean grey value of the TH signal was measured in different areas within the neuron. Next, fluorescence intensity at the soma, fluorescence intensities at different points along the axon and fluorescence intensity at the last point of the axon were compared.

3.9.4. Tracings

Using the Neuron J plugin of the Image J programme, the total neurite length, the number of neurites and the neurite type (primary, secondary or tertiary) were determined.

3.10 Statistical analysis

The statistical analyses of the results data were performed using ANOVA test (* $p<0.05$ ** $p<0.01$ *** $p<0.001$) with SEM error bars and were plotted using Prism (Mac OS X). Three independent experiments (n) were performed in all the analysis.

RESULTS

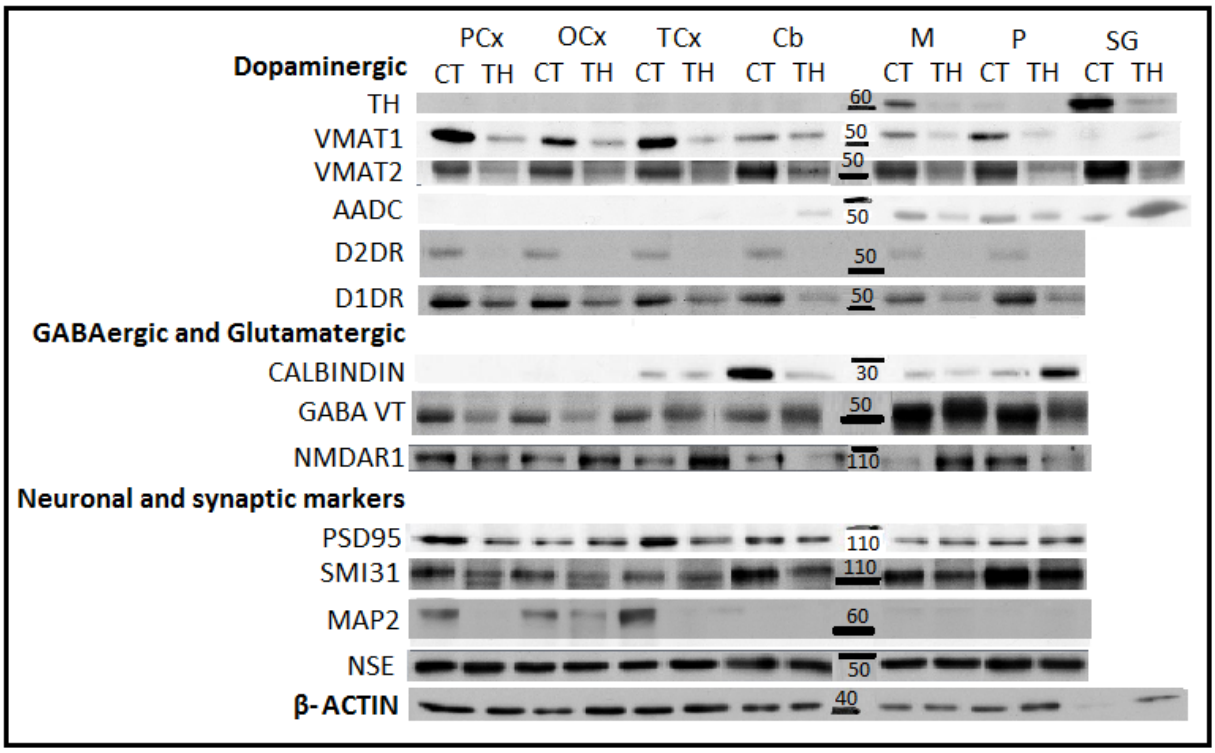
1. Study of a THD postmortem brain sample

In spite of existing studies, the neurobiological mechanisms underlying the THD variable clinical spectrum are poorly known. Since motor function, behaviour and learning processes can be affected in this disorder, it's plausible to hypothesize that THD pathophysiology is complex and involves different brain areas and functions.

Our aim with this study was to improve the knowledge of the pathophysiology of this disease through the study of different neurotransmitter pathways and proteins involved in neuronal development in a THD 16-week-old fetal brain carrying mutations (p. R328W and P. T399M) that caused a B phenotype in her sister (Møller et al. 2005).

This patient is a 20-year-old girl that was diagnosed at 3 years of age due to severe parkinsonism and very low CSF HVA levels; confirmed later by TH gene screening. In spite of being treated with L-dopa and other dopaminergic agonists, the patient suffers from severe intellectual disability, with a marked absence of expressive language, other communication skills and personal autonomy habits.

Figure 7: Western Blot bands of the proteins studied:



Proteins expression was assessed in different brain regions (Parietal (PCx), Occipital (OCx), Temporal Cortex (TCx), Cerebellum (Cb),

Mesencephalon (M) and Pons (P)). Some were analyzed also in the Suprarenal Gland (SG). The results (TH) were compared to an age-matched control (CT).

1.1 Dopaminergic neurotransmitter system proteins expression

Different dopaminergic proteins expression was assessed in order to understand how this neurotransmitter pathway, the most involved in THD, was affected.

We studied the expression of Tyrosine Hydroxylase (TH), which catalyses the rate limiting step in the biosynthesis of DA. The Vesicular monoamine transporters 1 (VMAT1) and 2 (VMAT2) that mediate monoamine loading in the vesicles. Aromatic-L-amino acid-decarboxylase (AADC) which catalyses the second step in the biosynthesis of DA, converting the L-Dopa to DA and Dopamine receptors 1 (D1DR) and 2 (D2DR), since the binding of DA to its receptors regulates specific neuronal circuits involved in movement disorders.

TH, VMAT1 and VMAT2 were under-expressed in all the brain regions and in the SG in the patient, when compared to the control brain. AADC was over-expressed in patient's cerebellum and SG. Dopamine receptors were under-expressed in all brain regions of the patient, the D2DR being the most severely under-expressed when compared to the control.

1.2 Glutamatergic neurotransmitter system proteins expression

To study Glutamatergic neurotransmitter systems different NMDA receptor markers were assessed but finally we were only able to measure N-methyl-D-aspartate receptor 1 (NMDAR1) expression, a receptor where glutamate binds.

NMDAR1 was over-expressed in mesencephalon and pons but under-expressed in parietal cortex compared to the control.

1.3 GABAergic neurotransmitter system proteins expression

We studied the following GABAergic proteins: Calbindin which is a GABAergic neuronal marker and GABA vesicular transporter (GABAVT), which mediates the package of GABA in the vesicles.

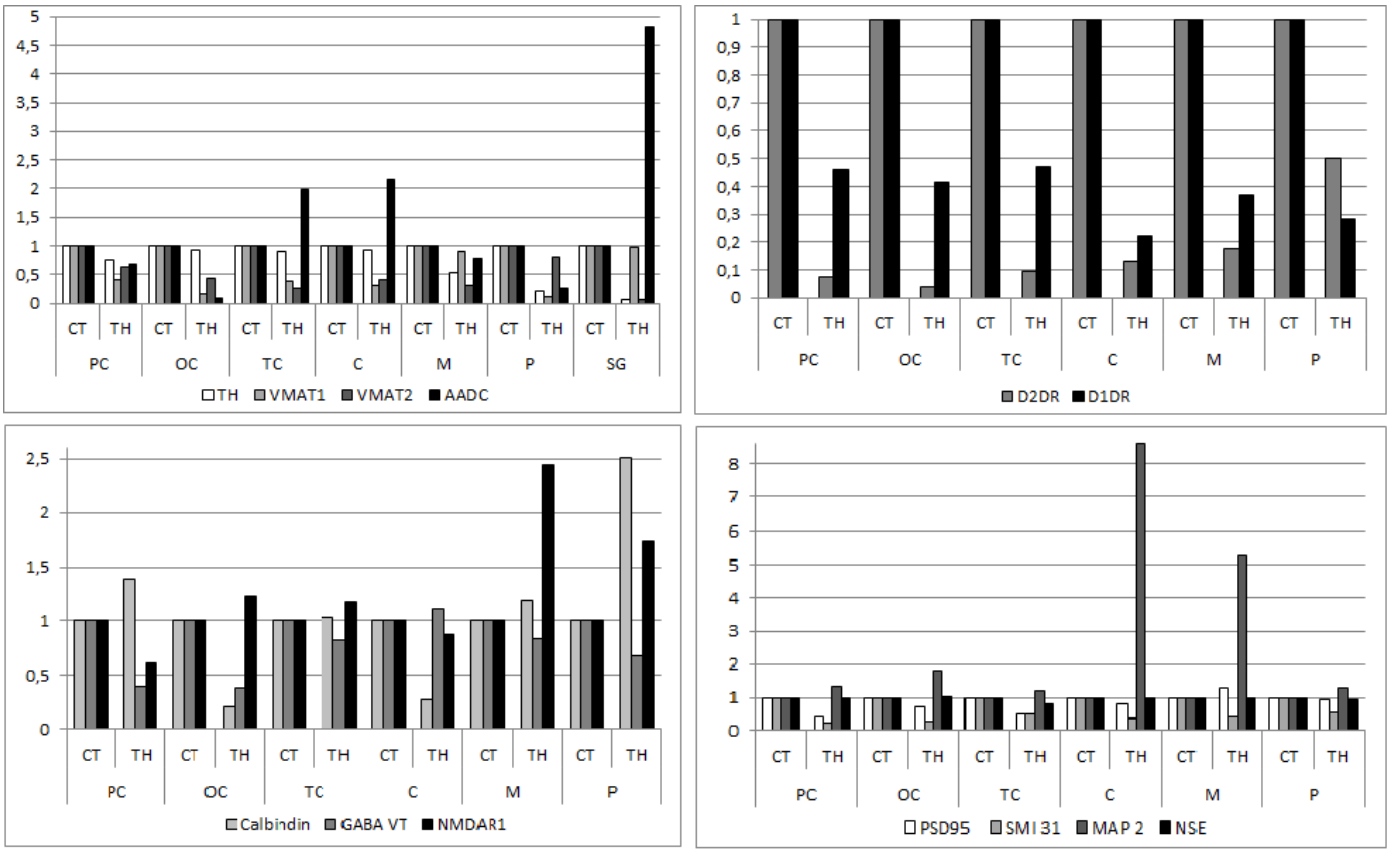
Calbindin was under-expressed in the cerebellum and overexpressed in the pons in the patient when compared to the control. A reduction of the GABAVT was observed in all the brain sections of the patient when compared to the control.

1.4 Expression of proteins involved in Central Nervous System (CNS)

Different neurodevelopmental biomarkers were studied to assess how general central nervous system development was affected. Postsynaptic density protein 95 (PSD95) was used as a synapse marker; NF-H (phosphorylated Neurofilament H) as an indicator of axons and neurons' soma; Microtubule-associated protein 2 (MAP2) for the somatodendritic compartment and Neuron-specific enolase (NSE) as a neuronal volume marker.

PSD95, NF-H and MAP2 were mostly under-expressed in all the patient's brain sections. No differences were observed in NSE expression when comparing both individuals.

Figure 8: Optic density measurement of protein expression:



The studied proteins were grouped as belonging to the dopaminergic pathway (graph 1 and 2), to the GABAergic and glutamatergic pathways (graph 3) and a group of neurodevelopmental markers (graph 4).

Table 12: Schematic differences of protein expression between both individuals:

	Region							
Protein	**PC**	**OC**	**TC**	**C**	**M**	**P**	**SG**	**MEAN**
TH	-	-	-	-	↓	↓	↓	↓ (27%)
VMAT1	↓	↓	↓	↓	↓	↓	↓	↓ (36%)
VMAT2	↓	↓	↓	↓	↓	↓	↓	↓ (33%)
AADC	-	-	-	↑	↓	↓	↑	↑ (121%)
D2DR	↓	↓	↓	↓	↓	↓	NT	↓ (12%)
D1DR	↓	↓	↓	↓	↓	↓	NT	↓ (36%)
CALBINDIN	-	-	=	↓	=	↑	NT	↓ (80%)
GABA VT	↓	↓	↓	↓	↓	↓	NT	↓ (66%)
NMDAR1	↓	↑	↑	↓	↑	↑	NT	↑ (122%)
PSD95	↓	↓	↓	↓	↓	↓	NT	↓ (73%)
SMI 31	↓	↓	↓	↓	↓	↓	NT	↓ (39%)
MAP2	↑	↑	↑	↑	↑	↑	NT	↑ (122%)
NSE	=	=	=	=	=	=	NT	= (95%)

Different protein expression (percentage) between brain regions and SG when comparing the patient to the control. Legend: NT: Not tested; –: no detection; =: same expression in both; ↑: Increased expression in the patient; ↓: Decreased expression in the patient

1.5 Brain region specific expression of the studied proteins

Apart from the specific expression of these proteins in THD brain when compared to the control, we also observed a global pattern of differential expression between some brain regions and the SG in both individuals.

TH was only expressed in mesencephalon, pons and SG. AADC was expressed also in the cerebellum. Calbindin was expressed in temporal cortex, cerebellum, mesencephalon and pons. Finally, although GABA VT and MAP2 were expressed in all the sections, GABA VT presented higher expression in the mesencephalon and pons and MAP2 in the cortical structures.

Table 13: Schematic differences of protein expression between all the brain regions and the suprarenal gland:

	Region						
Protein	**PC**	**OC**	**TC**	**C**	**M**	**P**	**SG**
TH	-	-	-	-	++	+	+++
AADC	-	-	-	+	++	+	+++
CALBINDIN	-	-	+	+++	++	++	NT
GABA VT	++	++	++	++	+++	+++	NT

Differential expression of some proteins between different brain regions and the Suprarenal Gland. Legend: NT: not tested; –: no detection; +: low expression; ++: mid expression; +++: high expression

2. Study of CSF proteomics in neurotransmitter defects patients

2.1 Population of study

In table 14 there is a full description of the patients included in the study with the variables age, sex, disease, severity, mutation and biochemical values of the different markers. Finally if the sample were pre- or post- treatment and for how much time have been with treatment:

Table 14: Clinical information of the population of study

Code	Age (years) Dx/treatment	Age (years) at CSF sample	Sex	Disease	Severity	Mutation	Biochemical Values						Treatment
							HVA	HVA/ 5HIAA	5-HIAA	3-OMD	NP/BP	Others	
ATAG_01	3,000	3,000	F	TH deficiency	Severe	THD: p.R238W/p.T399W	20 (↓) (304-658)	0,08 (↓) (1,92-3,44)	248 (=) (106-316)	1,66 (↓) (3-64)	?	Folate (43,9) (↓)	PRE TREATMENT
ATAG_02	0,417	0,417	F	TH deficiency	Moderate	THD: p.L236P/p.L236P (abolishes a restriction site)	31 (↓) (354-1328)	0,11 (↓) (1,16-2,4)	270 (=) (217-1142)	5 (↓) (20-162)	9 (=)(9-34)/ 42 (=) (12-44)	MHPG (2) (↓) (30-124)	PRE TREATMENT
ATAG_03	2,000	2,333	F	TH deficiency	Moderate	THD: p.L236P/p.L236P (abolishes a restriction site)	50 (↓) (344-906)	0,25 (↓) (1,11-3,48)	197 (=) (170-490)	10 (=) (4-50)	9(=) (8-43)/ 29(=) (8-54)		PRE TREATMENT
ATAG_04	0,417	0,417	F	TH deficiency	Moderate	THD: p.L236P/p.L236P (abolishes a restriction site)	19 (↓) (354-1328)	0,08 (↓) (1,16-2,4)	235 (=) (217-1142)	10 (↓) (20-162)	9 (=)(9-34)/35 (=) (12-44)	MHPG (1,4) (↓) (30-124)	PRE TREATMENT
ATAG_05	8,000	8,250	F	TH deficiency	Moderate	THD: p.L236P/p.L236P (abolishes a restriction site)	5 (↓) (202-596)	0,03 (↓) (1,20-3,45)	192(=) (87-366)	undetectable (↓) (5-60)	11(=)(10-46)/20(=)(8,2-68)	MHPG undetectable (↓) (13-68)	PRE TREATMENT
ATAG_06	0,917	0,917	F	TH deficiency	Mild	THD: p.R233H/p.R233H	158 (↓) (344-906)	0,64 (↓) (1,11-3,48)	248 (=) (170-490)	11 (=) (4-50)	7 (↓)(8-43)/ 34(=)(8-54)	-	PRE TREATMENT

ATAG_07	25,000	30,000	M	TH deficiency	Mild	THD: p.R233H/p.R233H	23 (↓) (156-410)	0,58 (↓) (1,44-3,17)	40 (↓) (63-185)	1 (=) (3-54)	18 (=)(10-24)/9 (↓)(13,7-36)	MHPG (5) (↓) (11-46)	POST TREATMENT (SEVERAL YEARS)
ATAG_08	16,000	17,333	M	TH deficiency	Mild	THD: p.R233H/p.R233H	53 (↓) (156-410)	0,73 (↓) (1,44-3,17)	73 (=) (63-185)	1 (↓) (3-54)	15(=)(10-24) /19(=)(13,7-36)	MHPG (7) (↓) (11-46)	POST TREATMENT (SEVERAL YEARS)
ATAG_09	11,000	11,500	F	TH deficiency	Severe	THD: p.R238W/p.T399W	285 (=) (156-410)	1,98 (=) (1,44-3,17)	144 (=) (63-185)	994 (↑) (3-54)	56 (↑) (10-24)/30 (=) (13,7-36)	MHPG (360) (↑) (11-46)	POST TREATMENT (SEVERAL YEARS)
ATAG_10	2,958	2,958	F	TH deficiency	Mild	THD: g-70C>T/g-70C>T??	118 (↓) (344-906)	0,89 (↓) (1,11-3,48)	132 (↓) (170-490)	295 (↑) (4-50)	44(↑)(8-43)/29 (=)(8-54)	-	POST TREATMENT (NOT KNOWN)
ATAG_11	6,000	6,750	F	TH deficiency	Mild	THD: p.R233H/p.R233H	651 (=) (304-658)	12,28 (↑) (1,92-3,44)	53 (↓) (analitical problem?) (106-316)	>6044 (↑) (3-64)	?	MHPG traces (↓) (22-54)/5-OHtrip traces (↓) (4-20)	POST TREATMENT (2 YEARS)
ATAG_12	0,583	0,583	F	TH deficiency	Moderate	THD: p.L236P/p.L236P (abolishes a restriction site)	18 (↓) (344-906)	0,07 (↓) (1,11-3,48)	247 (=) (170-490)	7 (=) (4-50)	11(=)(8-43)/43 (↑)(8-54)	MHPG (0) (↓) (20-80)	PRE TREATMENT
ATAG_13	15,000	15,900	F	GTPCH recessive	Mild	GTPCH: c.595C>G/c.595C>G	104 (↓) (156-410)	1,21 (↓) (1,44-3,17)	86 (=) (63-185)	466 (↑) (3-54)	3(↓)(10-24)/5 (↓)(13,7-36)	folato (MTHF) (↓) (39)/ PHE (↑) (80)	POST TREATMENT (SEVERAL YEARS)
ATAG_14	15,000	15,900	F	GTPCH recessive	Mild	GTPCH c.595C>G/c.595C>G	92 (↓) (156-410)	0,81 (↓) (1,44-3,17)	114 (=) (63-185)	230 (↑) (3-54)	2(↓)(10-24)/5 (↓)(13,7-36)	folato (MTHF) (↓) (33)/ PHE (↑) (62)	POST TREATMENT (SEVERAL YEARS)
ATAG_15	1,000	1,500	M	GTPCH recessive	Mild	recessive mutations in heterozygosis GCH1: c.671A>G (p.Lys224Arg) & c.403_411dupATAGACATG (p.Ile135_Met137dup)	120 (↓) (344-906)	1,62 (=) (1,11-3,48)	74 (↓) (170-490)	6 (=) (4-50)	86 (↑) (infection?)(8-43) / 4(↓)(8-54)	PHE (↑) (24)/MHPG (↓) (12) (20-80)/ GABA (↓) (28)	PRE TREATMENT

ATAG_16	1,000	1,580	M	GTPCH dominant	Mild	GTPCH: p.Q89X	268 (↓) (344-906)	1,14 (↓) (1,11-3,48)	236 (=) (170-490)	25 (=) (4-50)	2(↓)(8-43)/8(=)(8-54)	-	PRE TREATMENT
ATAG_17	7,000	7,000	F	GTPCH dominant	Moderate	GTPCH: p.Q89X	318 (=) (202-596)	1,84 (=) (1,20-3,45)	173 (=) (87-366)	60 (=) (5-60)	3(↓)(10-46)/8(↓) (8,2-68)	-	POST TREATMENT (1 YEAR)
ATAG_18	25,000	25,000	M	GTPCH dominant	Mild	?	75 (↓)(156-410)	0,97 (↓) (1,44-3,17)	77 (=)(63-185)	7 (=) (3-54)	5(↓)(10-24)/2 (↓) (13,7-36)	MHPG (9) (↓) (11-46)	?
ATAG_19	23,000	23,000	F	GTPCH dominant	Mild	?	28 (↓)(156-410)	2 (=) (1,44-3,17)	14 (↓)(63-185)	2 (=) (3-54)	5(↓)(10-24)/3 (↓) (13,7-36)	-	POST TREATMENT (20 YEARS)
ATAG_20	9,000	9,100	F	DHPR deficiency	Severe	?	531 (=) (202-596)	2,87 (=) (1,20-3,45)	185 (=) (87-366)	1210 (↑) (5-60)	30 (=)(10-46)/67(=) (8,2-68)	PHE (↑) (28)	POST TREATMENT (9 YEARS)
ATAG_21	19,000	19,100	M	PTPS deficiency	Moderate	?	14 (↓)(156-410)	14 (↑) (1,44-3,17)	1 (↓) (63-185)	9 (=) (3-54)	145 (↑)/5 (↓)(13,7-36)	PHE (↑) (65)/5OHT (traces, indicis)	POST TREATMENT (6 YEARS)
ATAG_22	16,000	16,333	M	PTPS deficiency	Moderate	?	33 (↓) (156-410)	33 (↑) (1,44-3,17)	1 (↓) (63-185)	13 (=) (3-54)	109 (↑) (10-24)/5 (↓)(13,7-36)	MHPG (5) (↓) (11-46)	POST TREATMENT (3 YEARS)
ATAG_23	15,000	15,000	M	DAT deficiency	Severe	SLC6A3:c.1400 G>T (p.Gly467Val) in homozygosis (hemizigosi),	534 (↑) (156-410)	24.27 (↑) (1,44-3,17)	22 (↓) (63-185)	38 (=) (3-54)	38 (↑) (10-24)/11 (↓)(13,7-36)	-	PRE TREATMENT
ATAG_24	13,000	13,916	M	AADC deficiency	Mild	DDC: c.206C>T y c.823G>A alterde protein (p.Thr69Met y p.Ala275Thr).	22 (↓) (156-410)	(↓) (1,44-3,17)	traces (↓) (63-185)	1391 (↑) (3-54)	15 (=) (10-24)/11 (↓)(13,7-36)	MHPG (traces) (↓) (11-46)	POST TREATMENT (8 YEARS)
ATAG_25	19,000	19,400	M	AADC deficiency	Mild	DDC: c.206C>T y c.823G>A alterde protein (p.Thr69Met y p.Ala275Thr).	3 (↓)(156-410)	traces (↓) (1,44-3,17)	traces (↓) (63-185)	696 (↑)(3-54)	15 (=)(10-24)/11(↓)(13,7-36)	B6 (24) (↓)/MHPG (traces) (↓) (11-46)	POST TREATMENT (8 YEARS)
ATAG_26	3,500	3,500	F	TH deficiency	Severe	TH:p? c.-71C>T/c.-71C>T TH deficiency	61,5 (↓) (211-871)	0,6 (↓) (1,5-3,5)	110 (=) (105-299)	11 (=) (<50)	9,4 (=)(9-30)/14,6 (=)(10-30)	-	PRE TREATMENT
ATAG_27	0,417	0,667	M	AADC deficiency	Severe	DDC: p.Val60Ala/p.Val60Ala c.179T>C/C.17	83,9 (↓) (310-1100)	0,8 (↓) (1,5-3,5)	102,4 (↓) (150-800)	1176 (↑) (<300)	19,5 (=)(15-35)/29,5 (=)(20-70)	-	PRE TREATMENT

						9T>C AADC deficiency							
ATAG_28	4,417	6,000	M	DHPR deficiency	Mild to Moderate	QDPR (gene) : p.Pro172Leu/p.Phe212Ser c.515C>T/c.635T>C DPHR deficiency	84,2 (↓) (211-871)	6,2 (↑) (1,5-3,5)	13,5 (↓) (105-299)	61 (↑) (<50)	20(=)(9-30)/ 26(=)(10-30)	-	PRE TREATMENT
ATAG_29	0,667	0,667	M	PTPS deficiency	Severe	PTS: p.Thr67Met/p.THr67Met c.200C>T/c.200C>T PTPS deficiency	155 (↓) (295-932)	1,9 (=) (1,5-3,5)	83,5 (↓) (114-336)	11 (=) (<100)	150(↓)(12-30)/3,3(↓)(15-40)	-	PRE TREATMENT
ATAG_30	14,000	14,750	F	GTPCH recessive	Severe	GCH1: p.Met213Ile/p.Met213Ile c.639G>A/c.639G>A GTPCH deficiency	30,3 (↓) (133-551)	1,2 (↓) (1,5-3,5)	24,4 (↓) (74-163)	4,8 (=) (<50)	0,3(↓)(9-20)/1,3(↓)(10-30)	-	PRE TREATMENT
ATAG_31	0,167	0,167	F	SR deficiency	?	SPR: p.Arg219*/p.Arg219* c.655C>T/c.655C>T SR deficiency	448 (=) (310-1100)	0,8 (↓) (1,5-3,5)	576 (=) (150-800)	75,1 (=) (<300)	22,5(=)(15-35)/37,8(=)(20-70)	-	?
ATAG_32	0,013	2,666666667	F	PTPS deficiency	Mild	PTPS: L26F/T67M	289 (↓) (364-870)	1.6 (=) (1,6-3.9)	181 (=) (155-359)	?	136 (↑)/ (5-53)/ ?	-	POST TREATMENT (2,5 YEARS)
ATAG_33	0,5	7	F	PTPS deficiency	Severe	PTPS: Thr106Met, Glu143Lys	256 (↓) (260-713)	9.8 (↑)(1,8-4,1)	26 (↓) (31-130)	?	?	-	POST TREATMENT (6 YEARS)
ATAG_34	0,1534	22	F	PTPS deficiency	Mild	PTPS: c.296A>G (Thy99Cys) hemizygous	?	?	?	?	108 (↑) (10-31)/ ?	-	POST TREATMENT (21 YEARS)
ATAG_35	0,1150	0,25	F	PTPS deficiency	Moderate	PTPS: c.200C>T (T67M); C260C>T (P87L)	292 (=) (403-919)	9.1 (↑) (1.8-3.0)	32 (↓) (170-412	?	86 (↑) (7-31)/ ?	-	POST TREATMENT (1 MONTH)
ATAG_36	0,1150	1,25	F	PTPS deficiency	Moderate	PTPS: c.200C>T (T67M);	480 (=) (364-870)	2.28 (=)(1.6-3.9)	210 (=)(155-359)	?	116 (↑)(5-53) /?	-	POST TREATMENT (1 YEAR)

						C260C>T (P87L)							
ATAG_37	0,1150	1,25	F	PTPS deficiency	Moderate	PTPS: c.200C>T (T67M); C260C>T (P87L)	480 (=) (364-870)	2.28 (=)(1.6-3.9)	210 (=)(155-359)	?	116 (↑)(5-53) /?	-	POST TREATMENT (1 YEAR)
ATAG_38	2,833	5,916	M	TH deficiency	Severe	TH: c.385C>T (Arf129X), c.692G>C (Arg231Pro)	73 (↓) (313-824)	0.3 (↓) (1.5-4.1)	236 (=)(130-362)	?	?	-	POST TREATMENT (2 YEARS)
ATAG_39	2,833	5,916	M	TH deficiency	Severe	TH: c.385C>T (Arf129X), c.692G>C (Arg231Pro)	73 (↓) (313-824)	0.3 (↓) (1.5-4.1)	236 (=)(130-362)	?	?	-	POST TREATMENT (2 YEARS)
ATAG_40	6,750	8,333	M	SR deficiency	Moderate	SR:c.530G>C (p.R177P) homozygous	80 (↓) (260-713)	0.9 (↓) (1.8-4.1)	87 (↓)(110-247)	?	?	-	POST TREATMENT (7 YEARS)
ATAG_41	1,112	1,75	F	TH deficiency	Moderate	TH: c707T>C (p.Leu236Pro) homozygous	301 (↓) (364-870)	0.08 (↓) (1.6-3.9)	357 (=) (155-359)	?	8 (=)(5-53)/ ?	-	POST TREATMENT (1 YEAR)
ATAG_42	1,112	2,833	F	TH deficiency	Moderate	TH: c707T>C (p.Leu236Pro) homozygous	229 (↓) (364-870)	1.25 (↓) (1.6-3.9)	183 (=) (155-359)	?	58 (↑)(5-53)/ ?	-	POST TREATMENT (1 YEAR)
ATAG_43	0,0328	0,03	F	DHPR deficiency	Mild	DHPR: c.49G>C (ü.Gly17Arg), c661C>T (p.Arg221X)	260 (↓) (484-1446)	4.1 (↑) (1.0-2.6)	63 (↓) (302-952)	?	14 (=)(6-59)/ ?	-	PRE TREATMENT
ATAG_44	not known	0,666666667	M	PTPS deficiency	?	PTPS	65 (↓) (403-919)	1.2 (↓) (1,8-3,0)	55 (↓) (170-412)	?	444 (↑)(7-31) /?	-	PRE TREATMENT
ATAG_45	not known	16	M	DHPR deficiency	Moderate	DHPR	276 (=) (115-455)	1.1 (=) (1,1-3,7)	158 (=) (51-204)	?	21 (=)(10-31) / ?	-	PRE TREATMENT
ATAG_46	not known	18	M	PTPS deficiency	?	PTPS	114 (↓) (115-455)	2.15 (=) (1,1-3,7)	53 (=) (51-204)	?	94 (↑)(10-31) / ?	-	POST TREATMENT (NOT KNOWN)
ATAG_47	2,000	6,000	F	PTPS deficiency	Severe	PTPS: Thr106Met; Glu134Lys (not known on DANN level)	485 (=) (313-824)	12.44 (↑) (1.5-4.1)	39 (↓) (130-362)	?	607 (↑) (5-53)/ ?	-	POST TREATMENT (5 YEARS)
ATAG_48	1,583	4,333	F	DHPR deficiency	Severe	DHPR: p.S115L/ p.S115L	142 (↓) (313-824)	4.43 (↑) (1.5-4.1)	32 (↓) (130-362)	?	52 (=) (5-53)/?	-	POST TREATMENT (3 YEARS)

ATAG_49	2,916	4,833	F	TH deficiency	Moderate	TH: c.698C>G /Arg233His); c500T>A (Val167Glu)	168 (=)(130-362)	0.6 (↓) (1.5-4.1)	269 (=) (130-362)	?	12 (=) (5-53)/?	-	POST TREATMENT (2 YEARS)
ATAG_50	0,278	20	F	PTPS deficiency	Mild	PTPS mutation not known	264 (=)(115-455)	3.1 (=) (1.1-3.7)	86 (=)(51-204)	?	?	-	POST TREATMENT (19 YEARS)
ATAG_51	not known	18	F	PTPS deficiency	?	PTPS mutation not known	362 (↓) (403-919)	2.5 (=) (1.8-3.0)	142 (↓) (170-412)	?	102 (↑) (7-31)/?	-	POST TREATMENT (NOT KNOWN)
ATAG_52	not known	1,25	F	PTPS deficiency	?	PTPS mutation not known	469 (=) (364-870)	2.8 (=) (1.6-3.9)	165 (=) (155-359)	?	106 (↑)(5-53)/?	-	POST TREATMENT (NOT KNOWN)
ATAG_53	not known	?	M	PTPS deficiency	?	PTPS mutation not known	153 (↓) (403-919)	2.4 (=) (1.8-3.0)	63 (↓) (170-412)	?	87(↑)(7-31) /?	-	POST TREATMENT (NOT KNOWN)
ATAG_54	6,000	20,000	F	SR deficiency	Mild-Moderate	-	157 (=) (156-410)	2,21 (=) (1,44-3,17)	71 (=) (63-185)	1710 (↑) (3-54)	32 (↑)(10-24) /55 (↑)(13,7-36)	-	POST TREATMENT (12 YEARS)
ATAG_55	newborn	8,000	M	GTPCH recessive	Mild-Moderate	GCH1 p.V206A homozygous	278 (=) (218-852)	1,06 (↓) (1,9-3,8)	261 (=) (66-388)	1545 (↑) (<100)	?	-	POST TREATMENT (8 YEARS)
ATAG_56	newborn	10,000	M	PTPS deficiency	Moderate-Severe	PTPS homozygous for c.155A>G(p.Asn52Ser)	362 (=) (167-563)	1,29 (↓) (1,9-3,8)	279 (↑) (67-189)	2206 (↑) (<100)	?	-	POST TREATMENT (10 YEARS)
ATAG_57	0,250	0,333	F	AADC deficiency	Severe	DDC c.714+4A (IVS6+4A>T) homozygous AADC	23 (↓) (450-1132)	3,83 (↑) (1,3-3,1)	6 (↓) (179-711)	1748 (↑) (<100)	?	-	PRE TREATMENT
ATAG_58	1.7	3,39	M	TH deficiency	Mild	G698A/G698A	49 (↓) (384-769)	0,17 (↓) (1,8-4,4)	284 (↑) (110-265)	ND (=) (<10)	9,2 (=) / 8,9 (↓)	-	PRE TREATMENT
ATAG_59	1.08	2,10	M	TH deficiency	Mild	Del C291/G698A	97 (↓) (384-769)	0,59 (↓) (1,8-4,4)	164 (=) (110-265)	0 (↓) (14-35)	9 (↓) / 11 (↓)	-	PRE TREATMENT
ATAG_60	3.17	3,19	F	TH deficiency	Mild	G698A/G698A	39 (↓) (384-769)	0,17 (↓) (1,8-4,4)	229 (=) (110-265)	?	4,9 (↓) / 4,6 (↓)	-	PRE TREATMENT
ATAG_61	7.08	7,11	M	TH deficiency	Mild	CRE-71C>T/c.1159 C>A (L387M)	69 (↓) (384-769)	0,59 (↓) (1,8-4,4)	117 (=) (110-265)	5 (<50)	13,5 (=) / 21,8 (=)	-	PRE TREATMENT
ATAG_62	2.58	2,62	F	TH deficiency	Mild	G698A/G698A	110 (↓) (384-769)	0,41 (↓) (1,8-4,4)	269 (↑) (110-265)	ND (=) (<10)	9,3 (=) / 8,8 (↓)	-	PRE TREATMENT

ATAG_63	2.50	2,50	M	TH deficiency	Severe	T707C/T707C	6 (↓) (330-668)	0,04 (↓) (1,9-3,8)	160 (=) (109-214)	4 (↓) (10-42)	8 (↓) / 12 (↓)	-	PRE TREATMENT
ATAG_64	30.0	31,03	F	TH deficiency	Moderate	c.974T>G (p.Leu325Arg) in exon 9 en c.1234C>A (p.Gln412Lys) in exon 12	29 (↓) (87-372)	0,31 (↓) (1,2-3,1)	94 (=) (58-190)	6 (↓) (10-42)	9.8 (=) /9.5 (=)	-	POST TREATMENT (0,25 YEARS)
ATAG_65	13.5	13,42	M	TH deficiency	Severe	G698A/G721A	11 (↓) (330-668)	0,19 (↓) (1,9-3,8)	59 (↓) (109-214)	9 (↓) (10-42)	?	-	PRE TREATMENT
ATAG_66	3.2	3,02	M	TH deficiency	Severe	G1076T/G1076T	124 (↓) (346-716)	0,57 (↓) (2,3-4.0)	217 (=) (100-245)	376 (↑) (10-42)	?	-	POST TREATMENT (1 YEAR)
ATAG_67	8.2	8,01	F	TH deficiency	Severe	IVS11-24T>A/G698A	49 (↓) (346-716)	0,24 (↓) (2,3-4.0)	201 (=) (100-245)	265 (↑) (2-25)	?	-	POST TREATMENT (1 YEAR)
ATAG_68	0.99	0,94	M	PTPS deficiency	?	?	179 (↓) (488-664)	2,45 (=) (2,1-2,9)	73 (↓) (156-275)	13 (=) (<100)	?	-	PRE TREATMENT
ATAG_69	1.53	1,53	M	DHPR deficiency	?	?	69 (↓) (429-789)	9,86 (↑) (1,6-3,3)	7 (↓) (156-275)	11 (↓) (18-67)	9.9 (=) /33.8 (=)	-	?
ATAG_70	?	25,87	F	PTPS deficiency	?	?	25 (↓) (87-372)	6,25 (↑) (1,2-3,1)	4 (↓) (58-190)	3 (↓) (10-42)	77.0 (↑) /4.6 (↓)	-	POST TREATMENT (20 YEARS)
ATAG_71	?	0,02	F	DHPR deficiency	?	c.661C>T (R221X) heterozygous	184 (↑) (<20)	5,26 (↑) (0,8-1,9)	35 (↓) (383-1028)	56 (=) (<325)	?	-	?
ATAG_73	8.0	8,30	M	AADC deficiency	?	?	16 (↓) (330-668)	?	?	1031 (↑) (10-42)	?	-	?
ATAG_75	1.3	1,18	F	AADC deficiency	?	?	18 (↓) (429-789)	36 (↑) (1,6-3,3)	0,5 (↓) (156-275)	1349 (↑) (18-67)	122.1 (↑) /51.7 (=)	-	PRE TREATMENT
ATAG_76	2.1	2,00	F	AADC deficiency	?	?	19 (↓) (429-789)	cannot be calculated	ND (↓) (156-275)	1497 (↑) (18-67)	12.6 (=) /26.5 (=)	-	PRE TREATMENT
ATAG_77	22.6	24,12	M	AADC deficiency	Mild	G102S; c.387G>A/ G102S; c.387G>A	<2 (↓) (100-400)	cannot be calculated	<2 (↓) (40-170)	3352 (↑) (10-42)	?	-	PRE TREATMENT
ATAG_78	6.0	5,93	M	AADC deficiency	Severe	Arg462Pro/Arg462Pro	47 (↓) (346-716)	7,83 (↑) (2,3-4.0)	6 (↓) (100-245)	709 (↑) (10-42)	?	-	PRE TREATMENT
ATAG_79	3.8	3,74	M	AADC deficiency	Severe	Ser250Phe/Ser250Phe	197 (↓) (384-769)	10,94 (↑) (1,8-4,4)	18 (↓) (110-265)	3525 (↑) (18-67)	?	-	POST TREATMENT (NOT KNOWN)
ATAG_80	3.8	3,55	M	AADC deficiency	Severe	Ser250Phe/Ser250Phe	69 (↓) (384-769)	3,83 (=) (1,8-4,4)	18 (↓) (110-265)	683 (↑) (18-67)	?	-	POST TREATMENT (0,5 YEARS)

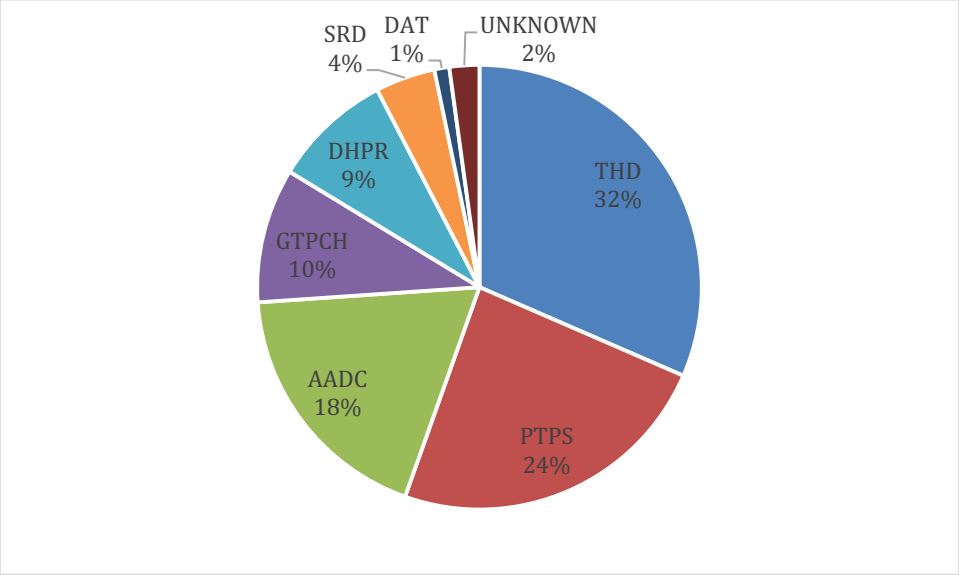
ATAG_81	0,410	0,46	M	AADC deficiency	Severe	Arg447His/Arg 447His	22 (↓) (478-895)	7,33 (↑) (1,3-3,1)	3 (↓) (231-618)	1585 (↑) (<100)	?	-	PRE TREATMENT
ATAG_82	1.9	1,71	F	AADC deficiency	?	?	11 (↓) (429-789)	3,67 (↑) (1,6-3,3)	3 (↓) (156-275)	589 (↑) (18-67)	15.6 (=) /33.9 (=)	-	PRE TREATMENT
ATAG_83	4,000	4,500	M	unknown -- GTPCH deficiency?	Mild	GTPCH deficiency is suspected: CNTNAP2 (NM_014141.5): c.2339C>T, p.Ser780Leu, heterozygosis, AR, TH (NM_199292.2): c.707T>C p.Leu236Pro heterozygosis	25.50 (↓) (304-658)	1,21 (↓) (1,92-3,44)	21.10 (↓) (106-316)	6.57 (=) (3-64)	23(=) (7-55)/31(=)(10-52)	-	PRE TREATMENT
ATAG_84	2,000	2,500	M	unknown-- pterines?	Moderate	mutation not known	56.00 (↓) (344-906)	1,19 (=) (1,11-3,48)	47.00 (↓) /170-490)	6.80 (=) (4-50)	42(=)(8-43)/51(=)(8-54)	-	PRE TREATMENT
ATAG_85	15,000	15,000	M	very complex (TH deficiency among others)	Moderate	TH: p.L236P/p.L236P c.707T>C/c.707T>C	65 (↓) (156-410)	2,03 (=) (1,44-3,17)	32 (↓) (63-185)	4 (=) (3-64)	18(=)(10-24)/23(=)(13,7-36)	-	PRE TREATMENT
ATAG_86	0,250	7,000	F	AADC deficiency	Severe	AADC: c.1040G>A in homozygosis (p.Arg347Gln); & mutation c.574T>C in homozygosis in the gen ARSB (Maroteaux-Lamy synd)	26 (↓) (350-1328)	1.63 (↓) (1,16-2,4)	16 (↓) (217-1142)	2281 (↑) (20-162)	?	-	PRE TREATMENT
ATAG_87	0,292	9,000	F	AADC deficiency	Severe	AADC: homozygous p.R347Q (c.1040G>A)	18 (↓) (350-1328)	(↑) (1,16-2,4)	traces (↓) (217-1142)	2239 (↑) (20-162)	?	-	POST TREATMENT (SEVERAL YEARS)
ATAG_88	0,013	19,900	F	SR deficiency	Moderate	SR c.448A>G and c.751A>T	167 (=)(145-324)	2,03 (↓)	82 (=) (67-140)	1827 (↑) (<100)	23 (=)(8-28)/13 (=)(10-30)	-	POST TREATMENT (SEVERAL YEARS)

ATAG_89	Birh	4,700	F	PTPS deficiency	Mild	PTPS c.155A>G (p.Asn52Ser) c.73C>G (p.Arg25Gly)	264 (=) (233-928)	1,12 (↓)	234 (=) (74-345)	1622 (↑) (<150)	115 (↑)(7-65)/10 (↓)(18-50)	-	POST TREATMENT (SEVERAL YEARS)
ATAG_90	0,038	3,600	F	PTPS deficiency	Mild	?	287(=) (233-928)	1,88 (↓)	153 (=) (74-345)	>2500 (↑) (<150)	124 (↑)(7-65)/11 (↓) (18-50)	-	POST TREATMENT (SEVERAL YEARS)
ATAG_91	Birh	44,700	M	PTPS deficiency	?	?	104 (↓)(145-324)	2,74 (↑)	38(↓)(67-140)	1264(↑)(<100)	81(8-28) (↑)/5 (↓)(10-30)	hyper phe at birth	POST TREATMENT (SEVERAL YEARS)
ATAG_92	Birh	6,900	F	DHPR deficiency	?	?	63 (↓)(218-852)	2,17 (↓)	29(↓)(66-388)	268(↑)(<100)	14 (↑)(7-40)/11 (=)(9-40)	hyper phe at birth	POST TREATMENT (SEVERAL YEARS)
ATAG_93	Birh	2,200	F	AADC deficiency	?	AADC DDC c.714+4A>T; c.286G>A p.Gly96Arg	<5 (↓)(233-928)	1 (↓)	<5 (↓) (74-345)	502(↑)(<150)	?	-	PRE TREATMENT
ATAG_94	Birh	20,600	M	AADC deficiency	Mild	?	24 (↓)(145-324)	1,14 (↓)	21(↓)(67-140)	286(↑)(<100)	?	-	PRE TREATMENT

This population of 92 patients with diverse neurotransmitter defects include: 29 TH, 17 AADC, 9 GTPCH, 8 DHPR, 22 PTPS, 4 SR and one DAT and 2 unknown patients with an average age of 6 years.

Of these patients, 39 were males and 51 females with a different range of severity. Of all these samples, 38 were pre-treatment, 48 post-treatment and 6 unknowns.

Figure 9: Graph representing the diseases in our population of study



There's only one dopamine transporter deficiency (DAT) patient, so it cannot be studied as the others, and only a descriptive analysis has been performed.

This patient is a male who has an homozygous mutation in SLC6A3 gene (c.1400 G>T; p. G467V), a severe disorder and has not received any treatment.

Regarding their biochemical values we observed as expected; an increase in HVA and a decrease in 5-HIAA and therefore an increase ratio of HVA/5-HIAA. Normal values of 3-OMD, increased NP and decreased BP levels.

He has different abundance in the proteins detected in this proteomic study but it was not possible to determine anything more.

2.2 Descriptive analysis

We performed a descriptive analysis of the biochemical markers values in our population of study.

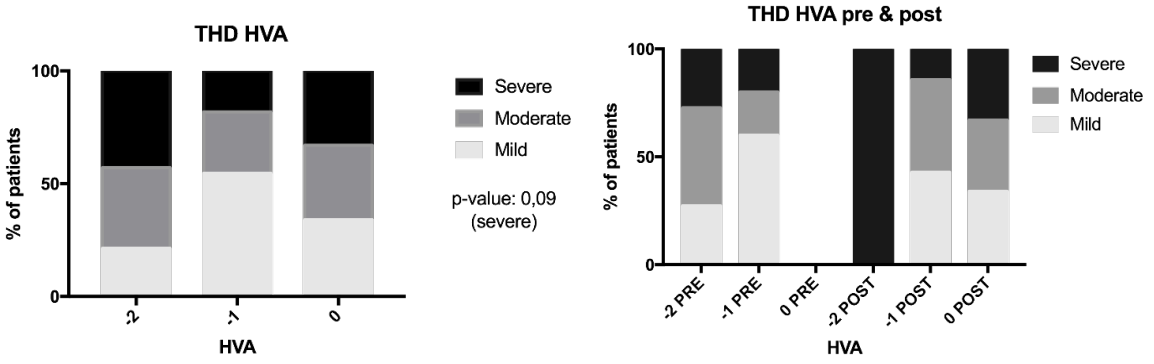
Table 15: Biochemical markers profile per disorder:

Disease	HVA	5-HIAA	HVA/5-HIAA	3-OMD	NP	BP
AADC	↓ in all patients	↓ in all patients	Highly variable	↑ in all patients	NA	NA
SRD	↓ or = in most of the patients	↓ or = in most of the patients	↓ or = in most of the patients	= in all patients	↑ or = in most of the patients	↑ or = in most of the patients
PTPS	Highly variable	Highly variable	Highly variable	NA	↑ in all patients	↓ in all patients
GTPCH	Highly variable	Highly variable	Highly variable	↑ or = in most of the patients	Highly variable	↓ or = in most of the patients
DHPR	Highly variable	Highly variable	Highly variable	Highly variable	↑ or = in most of the patients	= in all patients
TH	See graph below	See graph below	See graph below	Highly variable	Highly variable	Highly variable

Legend: NA: Not assessed; = Normal or maintained values; ↓: Decreased values; ↑ : Increased values.

Among these disorders, only in THD it was possible to observe a differential distribution of some BQ values by severity and response to treatment:

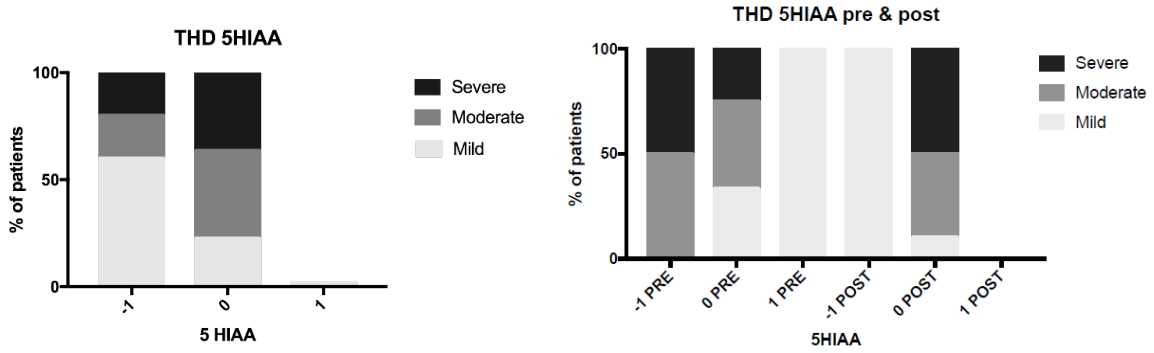
Figure 10: HVA levels in THD patients



There's a variable distribution of HVA levels among THD patients but it seems to be a differential distribution regarding severity. We can observe that the most severe patients have lower levels of HVA (-2) when compared to reference values even if it doesn't reach significance (p-value 0.09).

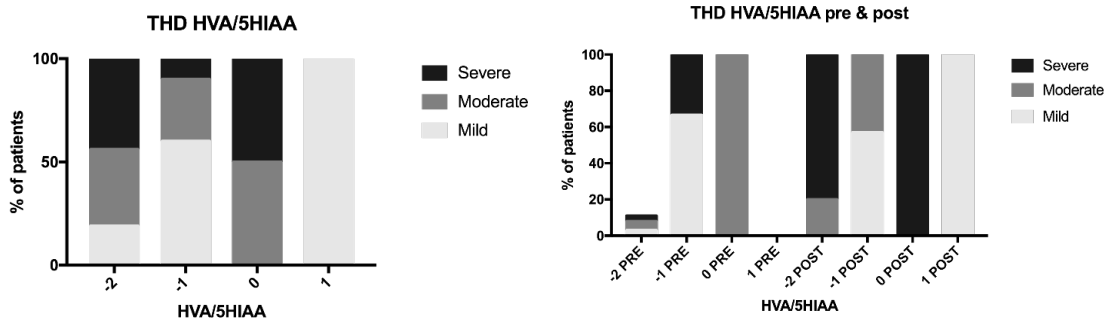
We could also observe how this distribution is also affected when we compare pre- and post-treatment samples. We can observe that severe patients after treatment continue to have low levels of HVA (-2) or the same levels whereas mild and moderate patients are able to increase or normalize those HVA levels.

Figure 11: 5-HIAA levels in THD patients



In all THD patients 5-HIAA levels were maintained or decreased and no clear distribution regarding severity or treatment was observed.

Figure 12: HVA/5-HIAA ratio levels in THD patients

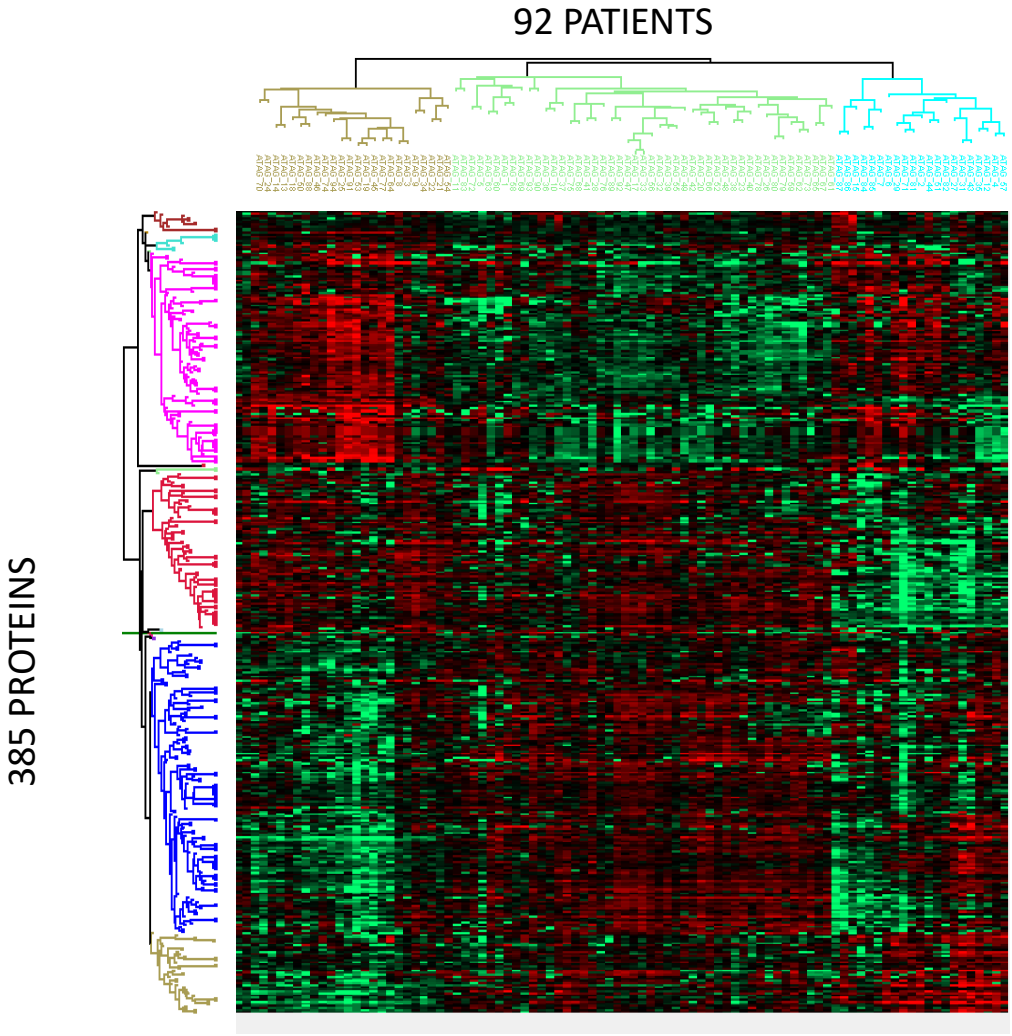


There's a highly variable distribution of HVA/5-HIAA levels among THD patients, even though only mild phenotypes have increased HVA/5-HIAA levels.

When compared pre and post-treatment samples we could observe that severe patients after treatment continue to have low levels of HVA/5HIAA (-2) or normalized levels whereas mild patients are able to maintain or increase those levels.

2.3 Detected proteins

Figure 13: Obtained matrix of proteins expression



After the proteomic study was performed in the 94 patients, we obtained a complete matrix of the proteins expression. In the case of this study we have 385 proteins expressed that are common in the 92 patients included in the study (2 were not taken into account due to quality issues).

2.4 Enrichment studies

As a general analysis, we observed that regardless the specific variables or disorders, a 15% of these common 385 proteins were related with nervous system development. This main category of common overrepresented proteins is related to crucial functions in neurodevelopment such as plasticity, excitability, synapse maturation and branching; and include glutamate receptors, axon guidance proteins and neurexins among others.

We then perform a bioinformatic analysis of the 385 set of proteins with Gene Ontology enrichment analysis and Panther analysis and we obtain the following categories overrepresented:

GO enrichment analysis for biological process:

- *NMDA glutamate receptor clustering* (7.43E-03)
- *PSD95 clustering* (1.04E-02)
- S*permine biosynthetic process* (3.61E-02)
- *Positive regulation of synapse maturation* (2.31E-02)

Panther analysis → "*Nervous system development"* enriched (1.57E-03)

2.5 Analysis per variables/diseases

We have analysed proteins abundance per different variables and diseases to see which ones were significatively represented and fulfil other analysis such as string network and functional enrichment analysis or just observed the box plot distribution.

The following variables/disorders were found of interest:

2.5.1 Disease

We obtained the following proteins with a signification (q- value < 0.001) in our population when disease variable and treatment were taken into account (in bold brain-related proteins):

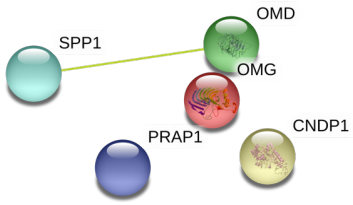
Table 16: Proteins significant per disease and treatment comparisons:

Protein	Name	Function	qtreatment
sp.Q96KN2.CNDP1_HUMAN	Beta-Ala-His dipeptidase	Preferential hydrolysis of the beta-Ala-|-His dipeptide (carnosine), and also anserine, Xaa-|-His dipeptides and other dipeptides including homocarnosine.	0.0002889
sp.Q99983.OMD_HUMAN	Osteomodulin	May be implicated in biomineralization processes. Has a function in binding of osteoblasts via the alpha(V)beta3-integrin (By similarity)	0.0002890
sp.P10451.OSTP_HUMAN	Osteopontin	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction.	0.0005001
sp.Q96NZ9.PRAP1_HUMAN	Proline-rich acidic protein 1	May play an important role in maintaining normal growth homeostasis in epithelial cells.	0.0005001

sp.P23515.OMGP_HUMAN	**Oligodendrocyte-myelin glycoprotein**	**Cell adhesion molecule contributing to the interactive process required for myelination in the central nervous system.**	**0.0007653**

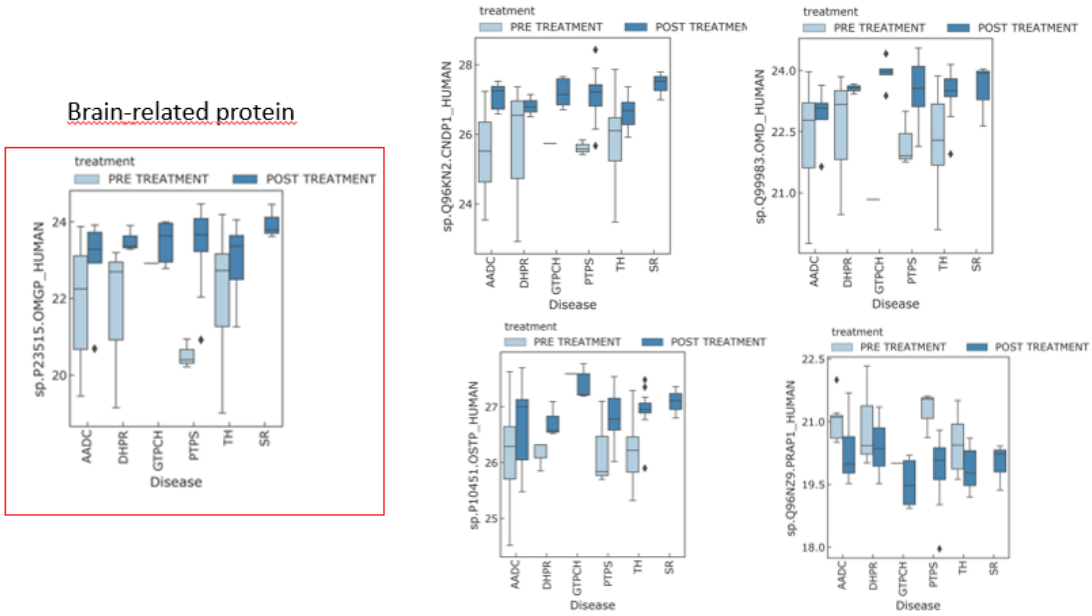
We performed string analysis to observe the network but we has only 5 nodes scarcely connected. Regarding functional enrichments in the network we found that regarding Gene ontology (Biological process) were: negative regulation of axonogenesis (FDR: 0.0227) and cell adhesion (FDR: 0.0453).

Figure 14: String analysis of variables disease - treatment



We plotted those significative proteins in a box plot graph to observe which proteins would be more interested to study. We selected for further analysis, OMGP as it is important in central nervous system and we observe how increased in post-treatment samples in the different disorders.

Figure 15: Box plots of variables disease – treatment



2.5.2 Severity

We obtained the following proteins with a signification (q- value < 0.001) in our population when severity variable and treatment were taken into account:

Table 17: Proteins significant per severity and treatment comparisons:

Protein	Name	Function	qtreatment
sp.P10451.OSTP_HUMAN	Osteopontin	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell-matrix interaction.	0.0009985
sp.Q99983.OMD_HUMAN	Osteomodulin	May be implicated in biomineralization processes. Has a function in binding of osteoblasts via the alpha(V)beta3-integrin.	0.0009986
sp.P23515.OMGP_HUMAN	**Oligodendrocyte-myelin glycoprotein**	**Cell adhesion molecule contributing to the interactive process required for myelination in the central nervous system.**	**0.0021606**
sp.Q96KN2.CNDP1_HUMAN	Beta-Ala-His dipeptidase	Preferential hydrolysis of the beta-Ala-|-His dipeptide (carnosine), and also anserine, Xaa-|-His dipeptides and other dipeptides including homocarnosine.	0.0033746
sp.P16870.CBPE_HUMAN	Carboxypeptidase E	Removes residual C-terminal Arg or Lys remaining after initial endoprotease cleavage during prohormone processing. Processes proinsulin.	0.0040787
sp.O15394.NCAM2_HUMAN	**Neural cell adhesion molecule 2**	**May play important roles in selective fasciculation and zone-to-zone projection of the primary olfactory axons.**	**0.0040787**
sp.Q96NZ9.PRAP1_HUMAN	Proline-rich acidic protein 1	May play an important role in maintaining normal growth homeostasis in epithelial cells.	0.0062808
sp.P17174.AATC_HUMAN	**Aspartate aminotransferase**	**Biosynthesis of L-glutamate from L-aspartate or L-cysteine. Important regulator of levels of glutamate, the major excitatory neurotransmitter of the vertebrate central nervous system. Acts as a scavenger of glutamate in brain neuroprotection.**	0.0083041
sp.P42785.PCP_HUMAN	Lysosomal Pro-X carboxypeptidase	Cleaves C-terminal amino acids linked to proline in peptides such as angiotensin II, III and des-Arg9-bradykinin. This cleavage occurs at acidic pH, but enzymatic activity is retained with some substrates at neutral pH.	0.0083041
sp.Q15818.NPTX1_HUMAN	**Neuronal pentraxin-1**	**May be involved in mediating uptake of synaptic material during synapse remodelling**	**0.0083041**

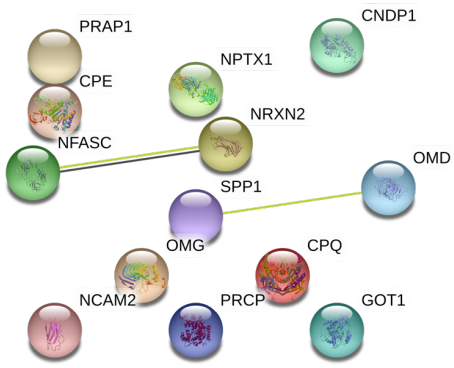
		or in mediating the synaptic clustering of AMPA glutamate receptors at a subset of excitatory synapses.	
sp.O94856.NFASC_HUMAN	**Neurofascin**	**Cell adhesion, ankyrin-binding protein which may be involved in neurite extension, axonal guidance, synaptogenesis, myelination and neuron-glial cell interactions.**	**0.0087224**
sp.Q9P2S2.NRX2A_HUMAN	**Neurexin-2**	**Neuronal cell surface protein that may be involved in cell recognition and cell adhesion. May mediate intracellular signalling.**	**0.0096490**
sp.Q9Y646.CBPQ_HUMAN	Carboxypeptidase Q	Carboxypeptidase that may play an important role in the hydrolysis of circulating peptides. Catalyses the hydrolysis of dipeptides with unsubstituted terminals into amino acids. May play a role in the liberation of thyroxine hormone from its thyroglobulin.	0.0098570

We performed string analysis to observe the network and we obtain 12 nodes scarcely connected. Regarding functional enrichments in the network we found the following categories:

Table 18: Functional enrichments' categories in the network disease- treatment

Category	Subcategory	False discovery rate (FDR)
GO (Biological process)	Cell Adhesion	0,0051
	Neuron-cell adhesion	0,0095
	Axon development	0,0129
GO (Cellular component)	Neuron Part	0,0485
KEGG Pathways	Cell Adhesion Molecules (CAMs)	0,0025

Figure 16: String analysis of variables severity- treatment

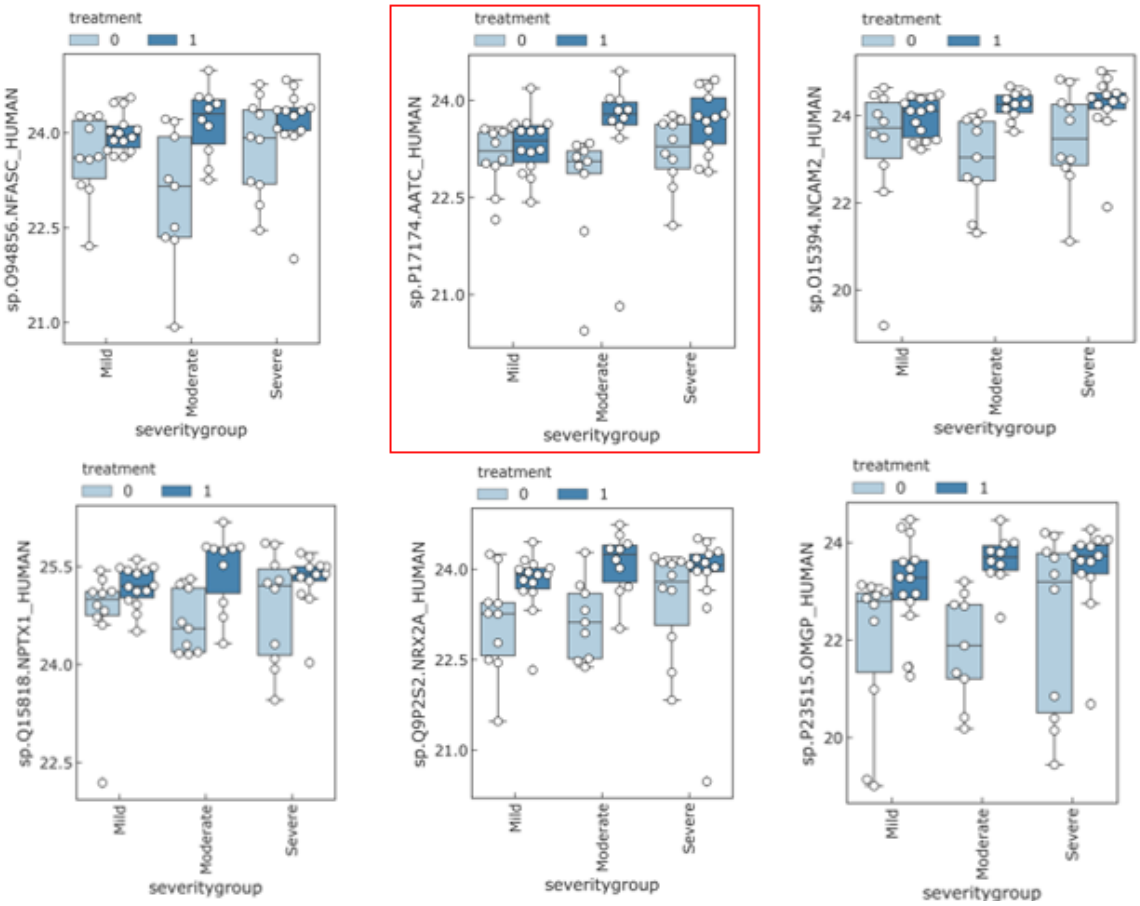


We plotted those 12 significative proteins in box plot graphs to observe which proteins had a clearer trend to increase or decrease with treatment. We split them in two groups: brain-related proteins or others.

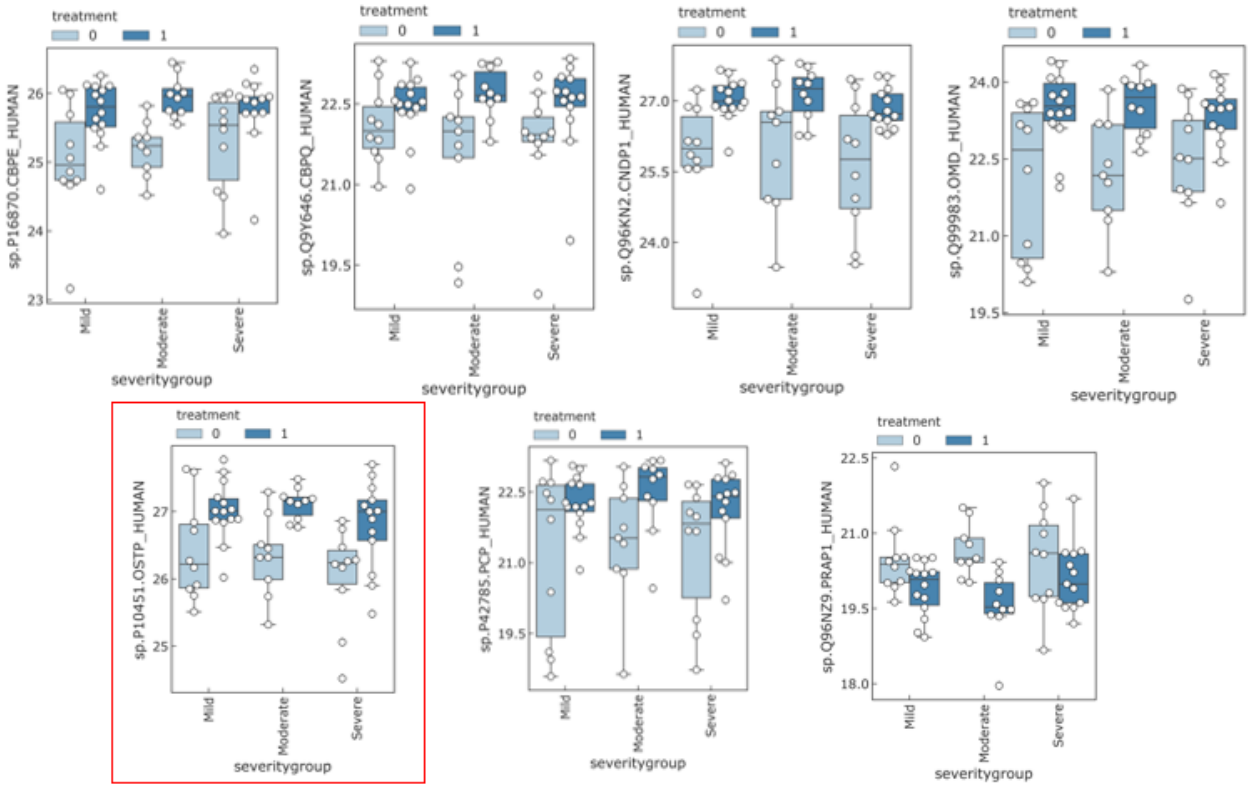
We selected for further analysis the brain-related proteins Aspartate Aminotransferase (AATC) as it regulates glutamate metabolism and increases after treatment in moderate and severe patients. Also, osteopontin which is important in cell-matrix alterations and which increases after treatment in all severity groups.

Figure 17: Box plots of variables severity – treatment

A) Brain related proteins



B) Other proteins



We then wanted to explore which proteins were altered in abundance in the different disorders present in our population:

2.5.3 Aromatic L-amino acid decarboxylase (AADC) deficiency

We found the following proteins (in bold brain-related proteins) with a signification (q- value < 0.05) in our AADC patients when severity variable was taken into account:

Table 19: Proteins significant in AADC disorder and severity:

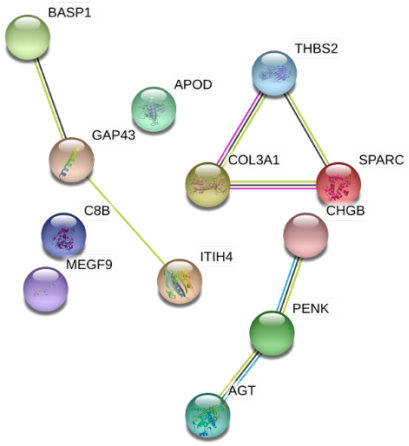
Protein	Name	Function	qseverity
sp.P01210.PENK_HUMAN	**Proenkephalin-A**	**Met- and Leu-enkephalins compete with and mimic the effects of opiate drugs. They play a role in a number of physiologic functions, including pain perception and responses to stress. PENK(114-133) and PENK(237-258) increase glutamate release in the striatum. PENK(114-133) decreases GABA concentration in the striatum.**	**0.0025270**
sp.P17677.NEUM_HUMAN	**Neuromodulin**	**This protein is associated with nerve growth. It is a major component of the motile "growth cones" that form the tips**	**0.0025271**

		of elongating axons. Plays a role in axonal and dendritic filopodia induction.	
sp.Q9H1U4.MEGF9_HUMAN	Multiple epidermal growth factor-like domains protein 9	Not known	0.0315476
sp.Q14624.ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	Type II acute-phase protein (APP) involved in inflammatory responses to trauma. May also play a role in liver development or regeneration.	0.0376579
sp.P80723.BASP1_HUMAN	Brain acid soluble protein 1	Membrane bound protein with several transient phosphorylation sites and PEST motifs. Conservation of proteins with PEST sequences among different species supports their functional significance. PEST sequences typically occur in proteins with high turnover rates. Immunological characteristics of this protein are species specific.	0.0376579
sp.P01617.KV204_HUMAN	Immunoglobulin kappa variable 2D-28	V region of the variable domain of immunoglobulin light chains that participates in the antigen recognition	0.0376579
sp.P09486.SPRC_HUMAN	SPARC	Appears to regulate cell growth through interactions with the extracellular matrix and cytokines. Binds calcium and copper, several types of collagen, albumin, thrombospondin, PDGF and cell membranes.	0.0376579
sp.P05060.SCG1_HUMAN	**Secretogranin-1**	**Secretogranin-1 is a neuroendocrine secretory granule protein, which may be the precursor for other biologically active peptides.**	**0.03801563**
sp.P06310.KV206_HUMAN	Immunoglobulin kappa variable 2-30	V region of the variable domain of immunoglobulin light chains that participates in the antigen recognition.	0.03878235
sp.P05090.APOD_HUMAN	Apolipoprotein D	APOD occurs in the macromolecular complex with lecithin-cholesterol acyltransferase. It is probably involved in the transport and binding of bilin. Appears to be able to transport a variety of ligands in a number of different contexts.	0.04545148
sp.P01019.ANGT_HUMAN	Angiotensinogen	Essential component of the renin-angiotensin system (RAS), a potent regulator of blood pressure, body fluid and electrolyte homeostasis.	0.04545148
sp.P02461.CO3A1_HUMAN	Collagen alpha-1(III) chain	Collagen type III occurs in most soft connective tissues along with type I collagen. Involved in regulation of cortical development. Is the major ligand of ADGRG1 in the developing brain and binding to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by coupling ADGRG1 to GNA13 and possibly GNA12.	0.04545148
sp.P07358.CO8B_HUMAN	Complement component C8 beta chain	Constituent of the membrane attack complex (MAC) that plays a key role in the innate and adaptive immune response by forming pores in the plasma membrane of target cells.	0.04545148

sp.P35442.TSP2_HUMAN	**Thrombospondin-2**	**Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Ligand for CD36 mediating antiangiogenic properties.**	**0.04545148**

We performed string analysis to check the network and we obtain 12 nodes quite connected. Regarding functional enrichments in the network we found the following category for Gene ontology (cellular component) enriched: “distal axon” (FDR: 0.0090).

Figure 18: String analysis of variables AADC-severity

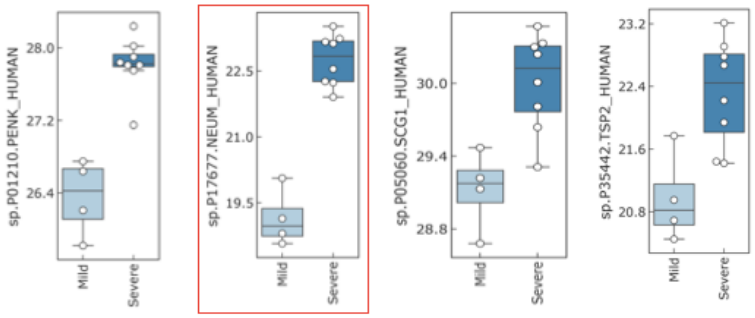


We plotted those significative proteins in box plot graphs to observe how these proteins increase or decrease with the more severe diagnosis in AADC patients. We split them in two groups: brain-related proteins or others.

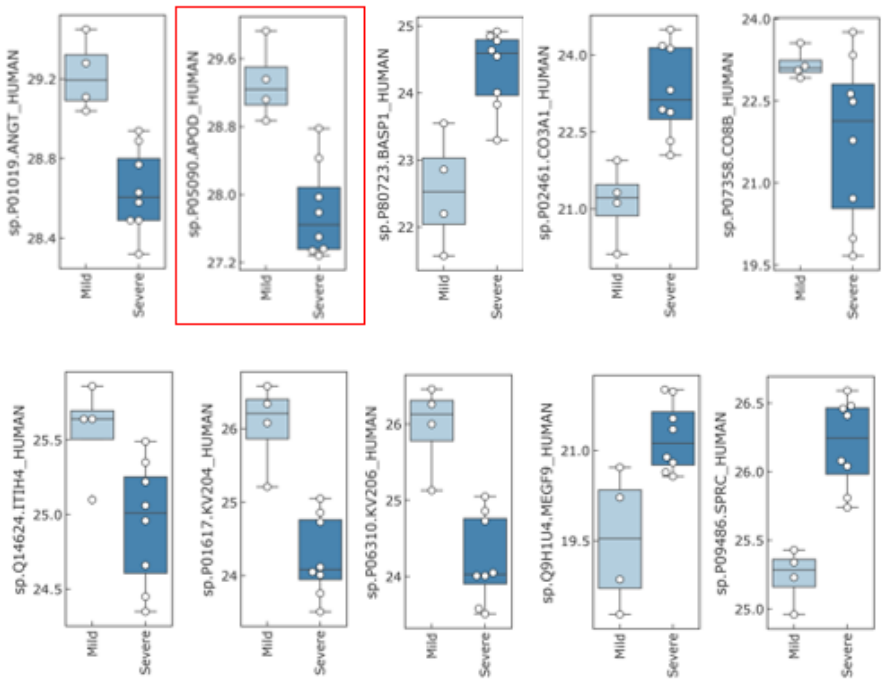
We selected for further analysis the brain-related protein neuromodulin (NEUM) as it is a major component of growth cones needed for axon elongation and is increased in severe patients. Also, Apolipoprotein D (APO D) which plays a role in the transport of a variety of compounds and decreases with severity.

Figure 19: Box plots of variables AADC –severity

A) Brain related proteins



B) Other proteins



2.5.4 BH4 disorders group

We then decided to group different disorders (GTPCH, SRD, PTPS & DHPR) in a group called BH4 disorders group as all of them are involved in the metabolism of BH4. It also presented the advantage of increasing the "n" and have more statistical power.

When taken all together we had the following significant proteins (in bold brain-related proteins) with a signification (q- value < 0.001) in our BH4 patients group when treatment was assessed:

Table 20: Proteins significant BH4 group disorders and treatment:

Protein	Name	Function	qtreatment
sp.P07585.PGS2_HUMAN	Decorin	May affect the rate of fibrils formation.	0.00013603
sp.P40189.IL6RB_HUMAN	**Interleukin-6 receptor subunit beta**	**Signal-transducing molecule. The receptor systems for IL6, LIF, OSM, CNTF, IL11, CTF1 and BSF3 can utilize IL6ST for initiating signal transmission. Binding of IL6 to IL6R induces IL6ST homodimerization and formation of a high-affinity receptor complex, which activates Janus kinases. That causes phosphorylation of IL6ST tyrosine residues which in turn activates STAT3. Mediates signals which regulate immune response, hematopoiesis, pain control and bone metabolism. Has a role in embryonic development. Does not bind IL6. Essential for survival of motor and sensory neurons and for differentiation of astrocytes. Required for expression of TRPA1**	**0.00020824**

		in nociceptive neurons. Required for the maintenance of PTH1R expression in the osteoblast lineage and for the stimulation of PTH-induced osteoblast differentiation.	
sp.P47972.NPTX2_HUMAN	**Neuronal pentraxin-2**	**Likely to play role in the modification of cellular properties that underlie long-term plasticity. Binds to agar matrix in a calcium-dependent manner**	**0.00047388**
sp.P26992.CNTFR_HUMAN	Ciliary neurotrophic factor receptor subunit alpha	Binds to CNTF. The alpha subunit provides the receptor specificity.	0.00047388
sp.Q96S96.PEBP4_HUMAN	Phosphatidylethanolamine-binding protein 4	Seems to promote cellular resistance to TNF-induced apoptosis by inhibiting activation of the Raf-1/MEK/ERK pathway, JNK and phosphatidylethanolamine externalization.	0.00047388
sp.Q9P2S2.NRX2A_HUMAN	**Neurexin-2**	**Neuronal cell surface protein that may be involved in cell recognition and cell adhesion. May mediate intracellular signaling.**	**0.00047388**
sp.P40925.MDHC_HUMAN	Malate dehydrogenase, cytoplasmic	Enzyme that catalyzes the NAD/NADH-dependent, reversible oxidation of malate to oxaloacetate in many metabolic pathways, including the citric acid cycle. Two main isozymes are known to exist in eukaryotic cells: one is found in the mitochondrial matrix and the other in the cytoplasm. This gene encodes the cytosolic isozyme, which plays a key role in the malate-aspartate shuttle that allows malate to pass through the mitochondrial membrane to be transformed into oxaloacetate for further cellular processes.	0.00047388
sp.P11362.FGFR1_HUMAN	**Fibroblast growth factor receptor 1**	**Tyrosine-protein kinase that acts as cell-surface receptor for fibroblast growth factors and plays an essential role in the regulation of embryonic development, cell proliferation, differentiation and migration. Required for normal mesoderm patterning and correct axial organization during embryonic development, normal skeletogenesis and normal development of the gonadotropin-releasing hormone (GnRH) neuronal system.**	**0.00073064**
sp.Q15818.NPTX1_HUMAN	**Neuronal pentraxin-1**	**May be involved in mediating uptake of synaptic material during synapse remodeling or in mediating the synaptic clustering of AMPA glutamate receptors at a subset of excitatory synapses.**	**0.00100004**
sp.P16870.CBPE_HUMAN	Carboxypeptidase E	Removes residual C-terminal Arg or Lys remaining after initial endoprotease cleavage during prohormone processing. Processes proinsulin.	0.00130009
sp.O75326.SEM7A_HUMAN	**Semaphorin-7A**	**Plays an important role in integrin-mediated signaling and functions both in regulating cell migration and immune responses.**	**0.00139504**

sp.O60888.CUTA_HUMAN	Protein CutA	May form part of a complex of membrane proteins attached to acetylcholinesterase (AChE).	0.00139504
sp.P06681.CO2_HUMAN	Complement C2	Component C2 which is part of the classical pathway of the complement system is cleaved by activated factor C1 into two fragments: C2b and C2a. C2a, a serine protease, then combines with complement factor C4b to generate the C3 or C5 convertase.	0.00157980
sp.Q99983.OMD_HUMAN	Osteomodulin	May be implicated in biomineralization processes. Has a function in binding of osteoblasts via the alpha(V)beta3-integrin	0.00157980
sp.P13591.NCAM1_HUMAN	**Neural cell adhesion molecule 1**	**This protein is a cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites among others.**	**0.00160091**
sp.Q9NYQ8.FAT2_HUMAN	**Protocadherin Fat 2**	**Involved in the regulation of cell migration. May be involved in mediating the organization of the parallel fibers of granule cells during cerebellar development.**	**0.00174535**
sp.Q16769.QPCT_HUMAN	Glutaminyl-peptide cyclotransferase	Responsible for the biosynthesis of pyroglutamyl peptides. Has a bias against acidic and tryptophan residues adjacent to the N-terminal glutaminyl residue and a lack of importance of chain length after the second residue. Also catalyzes N-terminal pyroglutamate formation. In vitro, catalyzes pyroglutamate formation of N-terminally truncated form of APP amyloid-beta peptides [Glu-3]-amyloid-beta. May be involved in the N-terminal pyroglutamate formation of several amyloid-related plaque-forming peptides.	0.00180063
sp.O95502.NPTXR_HUMAN	**Neuronal pentraxin receptor**	**May be involved in mediating uptake of synaptic material during synapse remodeling or in mediating the synaptic clustering of AMPA glutamate receptors at a subset of excitatory synapses**	**0.00182300**
sp.P42785.PCP_HUMAN	Lysosomal Pro-X carboxypeptidase	Cleaves C-terminal amino acids linked to proline in peptides such as angiotensin II, III and des-Arg9-bradykinin. This cleavage occurs at acidic pH, but enzymatic activity is retained with some substrates at neutral pH.	0.00182300
sp.O43556.SGCE_HUMAN	Epsilon-sarcoglycan	Component of the sarcoglycan complex, a subcomplex of the dystrophin-glycoprotein complex which forms a link between the F-actin cytoskeleton and the extracellular matrix.	0.00182300
sp.O15394.NCAM2_HUMAN	**Neural cell adhesion molecule 2**	**May play important roles in selective fasciculation and zone-to-zone projection of the primary olfactory axons.**	**0.00198458**
sp.P78324.SHPS1_HUMAN	**Tyrosine-protein phosphatase non-receptor type substrate 1**	**Immunoglobulin-like cell surface receptor for CD47. Acts as docking protein and induces translocation of PTPN6, PTPN11 and other binding partners from the cytosol to the plasma membrane. Supports adhesion of cerebellar neurons, neurite outgrowth and glial cell attachment. May play a key role in intracellular signaling during synaptogenesis and in synaptic**	**0.00205616**

		function. Involved in the negative regulation of receptor tyrosine kinase-coupled cellular responses induced by cell adhesion, growth factors or insulin. Mediates negative regulation of phagocytosis, mast cell activation and dendritic cell activation. CD47 binding prevents maturation of immature dendritic cells and inhibits cytokine production by mature dendritic cells	
sp.Q92876.KLK6_HUMAN	**Kallikrein-6**	**Serine protease which exhibits a preference for Arg over Lys in the substrate P1 position and for Ser or Pro in the P2 position. Shows activity against amyloid precursor protein, myelin basic protein, gelatin, casein and extracellular matrix proteins such as fibronectin, laminin, vitronectin and collagen. Degrades alpha-synuclein and prevents its polymerization, indicating that it may be involved in the pathogenesis of Parkinson disease and other synucleinopathies. May be involved in regulation of axon outgrowth following spinal cord injury. Tumor cells treated with a neutralizing KLK6 antibody migrate less than control cells, suggesting a role in invasion and metastasis.**	**0.00205616**
sp.Q562R1.ACTBL_HUMAN	Beta-actin-like protein 2	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.	0.00205616
sp.P23515.OMGP_HUMAN	**Oligodendrocyte-myelin glycoprotein**	**Cell adhesion molecule contributing to the interactive process required for myelination in the central nervous system.**	**0.00218232**
sp.Q92823.NRCAM_HUMAN	**Neuronal cell adhesion molecule**	**Cell adhesion protein that is required for normal responses to cell-cell contacts in brain and in the peripheral nervous system. Plays a role in neurite outgrowth in response to contactin binding. Plays a role in mediating cell-cell contacts between Schwa**	**0.00268866**
sp.O94856.NFASC_HUMAN	**Neurofascin**	**Cell adhesion, ankyrin-binding protein which may be involved in neurite extension, axonal guidance, synaptogenesis, myelination and neuron-glial cell interactions.**	**0.00268866**
sp.P04180.LCAT_HUMAN	Phosphatidylcholine-sterol acyltransferase	Central enzyme in the extracellular metabolism of plasma lipoproteins. Synthesized mainly in the liver and secreted into plasma where it converts cholesterol and phosphatidylcholines (lecithins) to cholesteryl esters and lysophosphatidylcholines on the surface of high and low density lipoproteins (HDLs and LDLs). The cholesterol ester is then transported back to the liver. Has a preference for plasma 16:0-18:2 or 18:O-18:2 phosphatidylcholines . Also produced in the brain by primary astrocytes, and esterifies free cholesterol on nascent APOE-containing lipoproteins secreted from glia and influences cerebral spinal fluid (CSF) APOE- and APOA1 levels. Together	0.00268866

		with APOE and the cholesterol transporter ABCA1, plays a key role in the maturation of glial-derived, nascent lipoproteins. Required for remodelling high-density lipoprotein particles into their spherical forms.	
sp.Q96KN2.CNDP1_HUMAN	Beta-Ala-His dipeptidase	Preferential hydrolysis of the beta-Ala-|-His dipeptide (carnosine), and also anserine, Xaa-|-His dipeptides and other dipeptides including homocarnosine.	0.00279948
sp.Q7Z3B1.NEGR1_HUMAN	**Neuronal growth regulator 1**	**May be involved in cell-adhesion. May function as a trans-neural growth-promoting factor in regenerative axon sprouting in the mammalian brain (By similarity).**	**0.00303229**
sp.Q6S8J3.POTEE_HUMAN	POTE ankyrin domain family member E	Not known	0.00324905
sp.P05067.A4_HUMAN	**Amyloid-beta precursor protein**	**Functions as a cell surface receptor and performs physiological functions on the surface of neurons relevant to neurite growth, neuronal adhesion and axonogenesis. Interaction between APP molecules on neighboring cells promotes synaptogenesis. Involved in cell mobility and transcription regulation through protein-protein interactions. Can promote transcription activation through binding to APBB1-KAT5 and inhibits Notch signaling through interaction with Numb. Couples to apoptosis-inducing pathways such as those mediated by G(O) and JIP. Inhibits G(o) alpha ATPase activity. Acts as a kinesin I membrane receptor, mediating the axonal transport of beta-secretase and presenilin 1. Involved in copper homeostasis/oxidative stress through copper ion reduction. In vitro, copper-metallated APP induces neuronal death directly or is potentiated through Cu2+-mediated low-density lipoprotein oxidation. Can regulate neurite outgrowth through binding to components of the extracellular matrix such as heparin and collagen I and IV. The splice isoforms that contain the BPTI domain possess protease inhibitor activity. Induces a AGER-dependent pathway that involves activation of p38 MAPK, resulting in internalization of amyloid-beta peptide and leading to mitochondrial dysfunction in cultured cortical neurons. Provides Cu2+ ions for GPC1 which are required for release of nitric oxide (NO) and subsequent degradation of the heparan sulfate chains on GPC1.**	**0.00336735**

sp.P78509.RELN_HUMAN	**Reelin**	**Extracellular matrix serine protease that plays a role in layering of neurons in the cerebral cortex and cerebellum. Regulates microtubule function in neurons and neuronal migration. Affects migration of sympathetic preganglionic neurons in the spinal cord, where it seems to act as a barrier to neuronal migration. Enzymatic activity is important for the modulation of cell adhesion. Binding to the extracellular domains of lipoprotein receptors VLDLR and LRP8/APOER2 induces tyrosine phosphorylation of DAB1 and modulation of TAU phosphorylation**	**0.00336735**
sp.P04406.G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	Has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities, thereby playing a role in glycolysis and nuclear functions, respectively. Participates in nuclear events including transcription, RNA transport, DNA replication and apoptosis. Nuclear functions are probably due to the nitrosylase activity that mediates cysteine S-nitrosylation of nuclear target proteins such as SIRT1, HDAC2 and PRKDC. Modulates the organization and assembly of the cytoskeleton. Facilitates the CHP1-dependent microtubule and membrane associations through its ability to stimulate the binding of CHP1 to microtubules (By similarity). Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate. Component of the GAIT (gamma interferon-activated inhibitor of translation) complex which mediates interferon-gamma-induced transcript-selective translation inhibition in inflammation processes. Upon interferon-gamma treatment assembles into the GAIT complex which binds to stem loop-containing GAIT elements in the 3'-UTR of diverse inflammatory mRNAs (such as ceruplasmin) and suppresses their translation.	0.00379302
sp.Q15223.PVRL1_HUMAN	**Nectin-1**	**Promotes cell-cell contacts by forming homophilic or heterophilic trans-dimers. Heterophilic interactions have been detected between NECTIN1 and NECTIN3 and between NECTIN1 and NECTIN4. Has some neurite outgrowth-promoting activity**	**0.00396969**
sp.P19021.AMD_HUMAN	Peptidyl-glycine alpha-amidating monooxygenase	Bifunctional enzyme that catalyses 2 sequential steps in C-terminal alpha-amidation of peptides. The monooxygenase part produces an unstable peptidyl(2-hydroxyglycine) intermediate that is dismutated to glyoxylate and the corresponding desglycine peptide amide by the lyase part. C-terminal amidation of peptides such as neuropeptides is essential for full biological activity.	0.00396969

sp.Q15904.VAS1_HUMAN	V-type proton ATPase subunit S1	Accessory subunit of the proton-transporting vacuolar (V)-ATPase protein pump, which is required for luminal acidification of secretory vesicles. Guides the V-type ATPase into specialized subcellular compartments, such as neuroendocrine regulated secretory vesicles or the ruffled border of the osteoclast, thereby regulating its activity. Involved in membrane trafficking and Ca2+-dependent membrane fusion. May play a role in the assembly of the V-type ATPase complex. In aerobic conditions, involved in intracellular iron homeostasis, thus triggering the activity of Fe2+ prolyl hydroxylase (PHD) enzymes, and leading to HIF1A hydroxylation and subsequent proteasomal degradation	0.00396969
sp.P09972.ALDOC_HUMAN	Fructose-bisphosphate aldolase C	In vertebrates, three forms of this ubiquitous glycolytic enzyme are found, aldolase A in muscle, aldolase B in liver and aldolase C in brain.	0.00413479
sp.Q99574.NEUS_HUMAN	**Neuroserpin**	**Serine protease inhibitor that inhibits plasminogen activators and plasmin but not thrombin. May be involved in the formation or reorganization of synaptic connections as well as for synaptic plasticity in the adult nervous system. May protect neurons from cell damage by tissue-type plasminogen activator**	**0.00413479**
sp.P04156.PRIO_HUMAN	**Major prion protein**	**Its primary physiological function is unclear. May play a role in neuronal development and synaptic plasticity. May be required for neuronal myelin sheath maintenance. May promote myelin homeostasis through acting as an agonist for ADGRG6 receptor. May play a role in iron uptake and iron homeostasis. Soluble oligomers are toxic to cultured neuroblastoma cells and induce apoptosis (in vitro).**	**0.00413479**
sp.P00751.CFAB_HUMAN	Complement factor B	Factor B which is part of the alternate pathway of the complement system is cleaved by factor D into 2 fragments: Ba and Bb. Bb, a serine protease, then combines with complement factor 3b to generate the C3 or C5 convertase. It has also been implicated in proliferation and differentiation of preactivated B-lymphocytes, rapid spreading of peripheral blood monocytes, stimulation of lymphocyte blastogenesis and lysis of erythrocytes. Ba inhibits the proliferation of preactivated B-lymphocytes.	0.00426014
sp.Q8N3J6.CADM2_HUMAN	**Cell adhesion molecule 2**	**Adhesion molecule that engages in homo- and heterophilic interactions with the other nectin-like family members, leading to cell aggregation. Important for synapse organization, providing regulated trans-synaptic adhesion. Preferentially binds to oligodendrocytes.**	**0.00463417**

sp.Q8TCZ2.C99L2_HUMAN	CD99 antigen-like protein 2	Plays a role in a late step of leukocyte extravasation helping cells to overcome the endothelial basement membrane. Acts at the same site as, but independently of, PECAM1. Homophilic adhesion molecule, but these interactions may not be required for cell aggregation.	0.00506299
sp.P98160.PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	Integral component of basement membranes. Component of the glomerular basement membrane (GBM), responsible for the fixed negative electrostatic membrane charge, and which provides a barrier which is both size- and charge-selective. It serves as an attachment substrate for cells. Plays essential roles in vascularization. Critical for normal heart development and for regulating the vascular response to injury. Also required for avascular cartilage development.	0.00530254
sp.P43121.MUC18_HUMAN	Cell surface glycoprotein MUC18	Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue. Its expression may allow melanoma cells to interact with cellular elements of the vascular system, thereby enhancing hematogeneous tumour spread. Could be an adhesion molecule active in neural crest cells during embryonic development. Acts as surface receptor that triggers tyrosine phosphorylation of FYN and PTK2/FAK1, and a transient increase in the intracellular calcium concentration.	0.00533689
sp.P17174.AATC_HUMAN	**Aspartate aminotransferase, cytoplasmic**	**Biosynthesis of L-glutamate from L-aspartate or L-cysteine. Important regulator of levels of glutamate, the major excitatory neurotransmitter of the vertebrate central nervous system. Acts as a scavenger of glutamate in brain neuroprotection. The aspartate aminotransferase activity is involved in hepatic glucose synthesis during development and in adipocyte glyceroneogenesis. Using L-cysteine as substrate, regulates levels of mercaptopyruvate, an important source of hydrogen sulfide. Mercaptopyruvate is converted into H2S via the action of 3-mercaptopyruvate sulfurtransferase (3MST). Hydrogen sulfide is an important synaptic modulator and neuroprotectant in the brain.**	**0.00634577**
sp.Q9UBP4.DKK3_HUMAN	**Dickkopf-related protein 3**	**Antagonizes canonical Wnt signaling by inhibiting LRP5/6 interaction with Wnt and by forming a ternary complex with the transmembrane protein KREMEN that promotes internalization of LRP5/6. DKKs play an important role in vertebrate development, where they locally inhibit Wnt regulated processes such as antero-posterior axial patterning, limb development, somitogenesis and eye formation. In the adult, Dkks are implicated in bone formation and bone disease, cancer and Alzheimer disease.**	**0.00634577**

sp.P04216.THY1_HUMAN	**Thy-1 membrane glycoprotein**	**May play a role in cell-cell or cell-ligand interactions during synaptogenesis and other events in the brain.**	**0.00744805**
sp.Q14515.SPRL1_HUMAN	SPARC-like protein 1	Calcium ion binding protein.	0.00755402
sp.P05543.THBG_HUMAN	Thyroxine-binding globulin (serpina 7)	There are three proteins including thyroxine-binding globulin (TBG), transthyretin and albumin responsible for carrying the thyroid hormones thyroxine (T4) and 3,5,3'-triiodothyronine (T3) in the bloodstream. This gene encodes the major thyroid hormone transport protein, TBG, in serum. It belongs to the serpin family in genomics, but the protein has no inhibitory function like many other members of the serpin family. Mutations in this gene result in TGB deficiency, which has been classified as partial deficiency, complete deficiency, and excess, based on the level of serum TBG.	0.00881832
sp.P12111.CO6A3_HUMAN	Collagen alpha-3(VI) chain	Collagen VI acts as a cell-binding protein.	0.00901392
sp.P60174.TPIS_HUMAN	Triosephosphate isomerase	Triosephosphate isomerase is an extremely efficient metabolic enzyme that catalyses the interconversion between dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (G3P) in glycolysis and gluconeogenesis.	0.00928896
sp.P07602.SAP_HUMAN	Prosaposin	Saposin-A and saposin-C stimulate the hydrolysis of glucosylceramide by beta-glucosylceramidase (EC 3.2.1.45) and galactosylceramide by beta-galactosylceramidase (EC 3.2.1.46). Saposin-C apparently acts by combining with the enzyme and acidic lipid to form an activated complex, rather than by solubilizing the substrate.	0.00998438

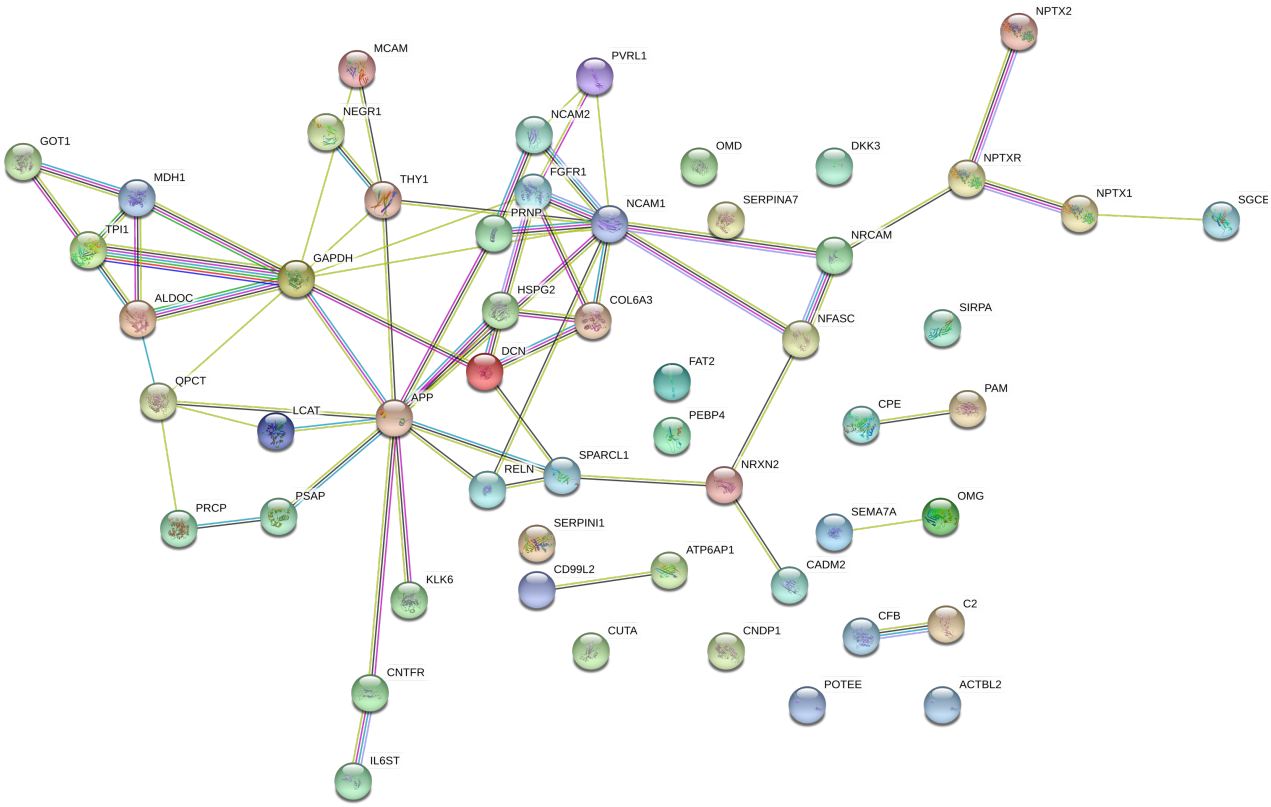
We performed string analysis to check the BH4 group network and we obtain 53 nodes highly connected among them. Regarding functional enrichments in the network we found the following categories enriched:

Table 21: Functional enrichments' categories in the network BH4 disorders- treatment

Category	Subcategory	False discovery rate (FDR)
GO (Biological process)	Cell Adhesion	7,66E-10
	Axon development	4,76E-05
	Cell-cell adhesion	7,43E-05
	Developmental Process	7,43E-05
GO (Molecular function)	Cell Adhesion molecule binding	2,14E-05
GO (Cellular component)	Neuron Part	5,53E-05
KEGG pathway	Cell Adhesion Molecules (CAMs)	9,12E-06
	Prion Diseases	0,0022
Reactome pathways	Neurofascin interactions	0,0074
	NCAM1 interactions	0,0074

Uniprot keywords	Cell Adhesion	4,59E-12

Figure 20: String analysis of variables BH4 group-treatment

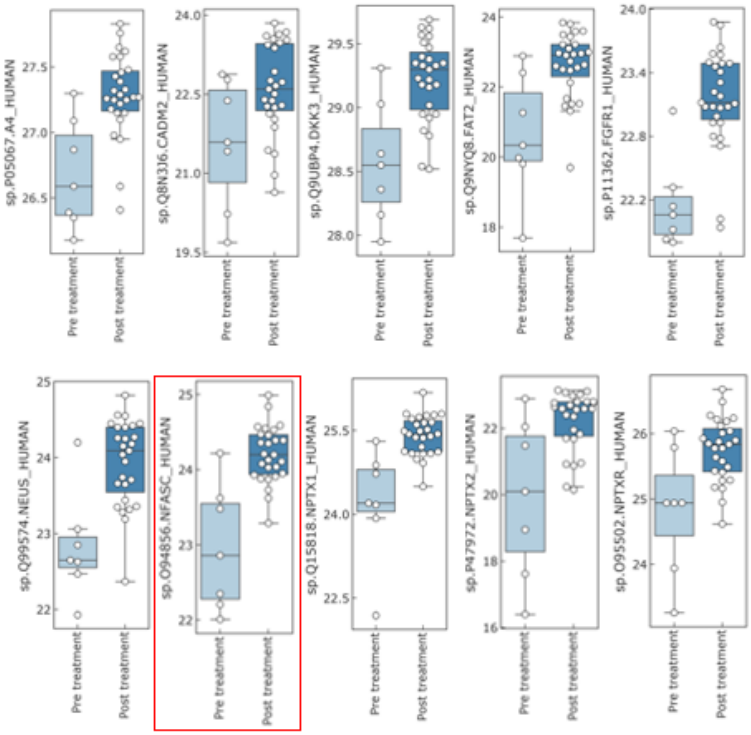


We plotted a selection of the significative proteins in box plot graphs to observe how these proteins increase or decrease with the treatment in BH4 group patients. We split them in two groups: brain-related proteins or others.

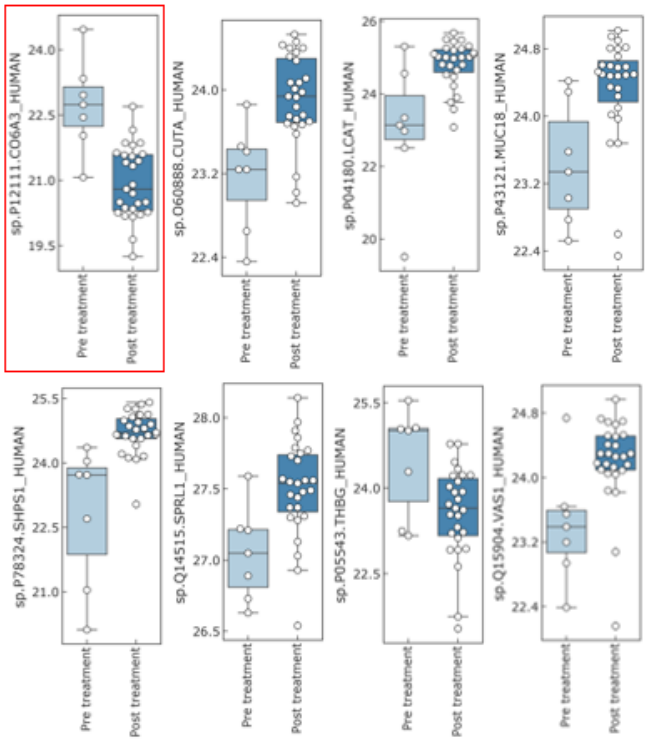
We selected for further analysis the brain-related protein neurofascin (NFASC) which may have different functions as neurite extension, axonal guidance or synaptogenesis among others and increases after treatment. Also, we selected Collagen alpha-3(VI) chain (COL6A3) which is involved in cell-binding and is decreased in post-treatment samples.

Figure 21: Box plots of variables BH4 – treatment

Brain related proteins



A) Other proteins



2.5.5 Tyrosine Hydroxylase deficiency (THD)

Then we focus on THD patients regarding severity and we found he following significant proteins (in bold brain-related proteins) with a signification (q- value < 0.005).

Table 22: Proteins significant in THD patients and severity:

Protein	Name	Function	qseverity
sp.P08697.A2AP_HUMAN	Alpha-2-antiplasmin	Serine protease inhibitor. The major targets of this inhibitor are plasmin and trypsin, but it also inactivates matriptase-3/TMPRSS7 and chymotrypsin.	0.00526314
sp.Q8N126.CADM3_HUMAN	Cell adhesion molecule 3	Involved in the cell-cell adhesion. Has both calcium-independent homophilic cell-cell adhesion activity and calcium-independent heterophilic cell-cell adhesion activity with IGSF4, NECTIN1 and NECTIN3. Interaction with EPB41L1 may regulate structure or function of cell-cell junctions.	0.03455358
sp.P07998.RNAS1_HUMAN	Ribonuclease pancreatic	Endonuclease that catalyses the cleavage of RNA on the 3' side of pyrimidine nucleotides. Acts on single-stranded and double-stranded RNA.	0.03455358
sp.Q15818.NPTX1_HUMAN	**Neuronal pentraxin-1**	**May be involved in mediating uptake of synaptic material during synapse remodelling or in mediating the synaptic clustering of AMPA glutamate receptors at a subset of excitatory synapses.**	**0.03455358**
sp.O60888.CUTA_HUMAN	Protein CutA	May form part of a complex of membrane proteins attached to acetylcholinesterase (AChE).	0.03455358
sp.P07602.SAP_HUMAN	**Prosaposin**	**Behaves as a myelinotrophic and neurotrophic factor, these effects are mediated by its G-protein-coupled receptors, GPR37 and GPR37L1, undergoing ligand-mediated internalization followed by ERK phosphorylation signaling.**	**0.03455358**
sp.P43121.MUC18_HUMAN	Cell surface glycoprotein MUC18	Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue. Its expression may allow melanoma cells to interact with cellular elements of the vascular system, thereby enhancing hematogeneous tumor spread. Could be an adhesion molecule active in neural crest cells during embryonic development. Acts as surface receptor that triggers tyrosine phosphorylation of FYN and PTK2/FAK1, and a transient increase in the intracellular calcium concentration.	0.03455358
sp.P13987.CD59_HUMAN	CD59 glycoprotein	Potent inhibitor of the complement membrane attack complex (MAC) action. Acts by binding to the C8 and/or C9 complements of the assembling MAC, thereby preventing incorporation of the multiple copies of C9 required for complete formation of the	0.03841871

		osmolytic pore. This inhibitor appears to be species-specific. Involved in signal transduction for T-cell activation complexed to a protein tyrosine kinase.	
sp.O94856.NFASC_HUMAN	**Neurofascin**	**Cell adhesion, ankyrin-binding protein which may be involved in neurite extension, axonal guidance, synaptogenesis, myelination and neuron-glial cell interactions.**	**0.04390508**
sp.P19021.AMD_HUMAN	Peptidyl-glycine alpha-amidating monooxygenase	Bifunctional enzyme that catalyses the post-translational modification of inactive peptidylglycine precursors to the corresponding bioactive alpha-amidated peptides, a terminal modification in biosynthesis of many neural and endocrine peptides.	0.04390508
sp.Q8TCZ2.C99L2_HUMAN	CD99 antigen-like protein 2	Plays a role in a late step of leukocyte extravasation helping cells to overcome the endothelial basement membrane. Acts at the same site as, but independently of, PECAM1. Homophilic adhesion molecule, but these interactions may not be required for cell aggregation.	0.04390508
sp.Q14982.OPCM_HUMAN	**Opioid-binding protein/cell adhesion molecule**	**Binds opioids in the presence of acidic lipids; probably involved in cell contact.**	**0.04390508**
sp.Q7Z3B1.NEGR1_HUMAN	**Neuronal growth regulator 1**	**May be involved in cell-adhesion. May function as a trans-neural growth-promoting factor in regenerative axon sprouting in the mammalian brain.**	**0.04390508**
sp.Q9UBS9.SUCO_HUMAN	SUN domain-containing ossification factor	Required for bone modelling during late embryogenesis. Regulates type I collagen synthesis in osteoblasts during their postnatal maturation.	0.04390508
sp.P02765.FETUA_HUMAN	Alpha-2-HS-glycoprotein	Promotes endocytosis, possesses opsonic properties and influences the mineral phase of bone. Shows affinity for calcium and barium ions.	0.04390508
sp.P23515.OMGP_HUMAN	**Oligodendrocyte-myelin glycoprotein**	**Cell adhesion molecule contributing to the interactive process required for myelination in the central nervous system.**	**0.04390508**
sp.Q96PD5.PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	May play a scavenger role by digesting biologically active peptidoglycan (PGN) into biologically inactive fragments. Has no direct bacteriolytic activity.	0.04390508
sp.P07333.CSF1R_HUMAN	**Macrophage colony-stimulating factor 1 receptor**	**Related with PD Tyrosine-protein kinase that acts as cell-surface receptor for CSF1 and IL34 and plays an essential role in the regulation of survival, proliferation and differentiation of hematopoietic precursor cells, especially mononuclear phagocytes, such as macrophages and monocytes.**	**0.04390508**
sp.P06727.APOA4_HUMAN	Apolipoprotein A-IV	May have a role in chylomicrons and VLDL secretion and catabolism. Required for efficient activation of lipoprotein lipase by ApoC-II; potent activator of LCAT. Apoa-IV is a major component of	0.04484336

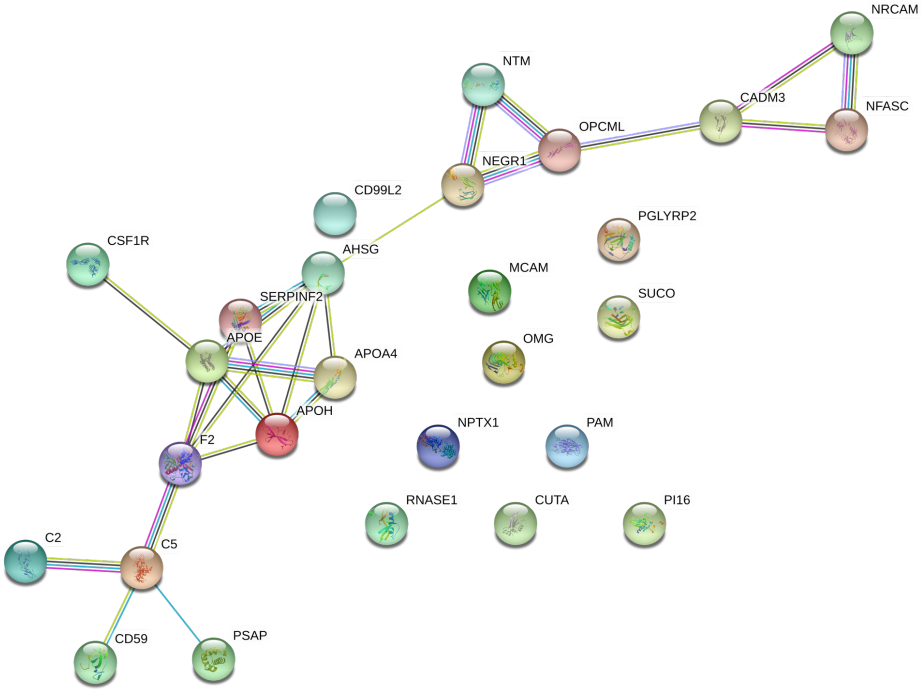
		HDL and chylomicrons.	
sp.P02649.APOE_HUMAN	Apolipoprotein E	APOE is an apolipoprotein, a protein associating with lipid particles, that mainly functions in lipoprotein-mediated lipid transport between organs via the plasma and interstitial fluids.	0.04484336
sp.Q92823.NRCAM_HUMAN	**Neuronal cell adhesion molecule**	**Cell adhesion protein that is required for normal responses to cell-cell contacts in brain and in the peripheral nervous system. Plays a role in mediating cell-cell contacts between Schwann cells and axons. Plays a role in the formation and maintenance of the nodes of Ranvier on myelinated axons. Nodes of Ranvier contain clustered sodium channels that are crucial for the saltatory propagation of action potentials along myelinated axons. During development, nodes of Ranvier are formed by the fusion of two heminodes. Required for normal clustering of sodium channels at heminodes; not required for the formation of mature nodes with normal sodium channel clusters. Required, together with GLDN, for maintaining NFASC and sodium channel clusters at mature nodes of Ranvier.**	**0.04484336**
sp.P02749.APOH_HUMAN	Beta-2-glycoprotein 1	Binds to various kinds of negatively charged substances such as heparin, phospholipids, and dextran sulfate. May prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells.	0.04484336
sp.P00734.THRB_HUMAN	Prothrombin	Thrombin, which cleaves bonds after Arg and Lys, converts fibrinogen to fibrin and activates factors V, VII, VIII, XIII, and, in complex with thrombomodulin, protein C. Functions in blood homeostasis, inflammation and wound healing.	0.04484336
sp.P06681.CO2_HUMAN	Complement C2	Component C2 which is part of the classical pathway of the complement system is cleaved by activated factor C1 into two fragments: C2b and C2a. C2a, a serine protease, then combines with complement factor C4b to generate the C3 or C5 convertase.	0.04648260
sp.P01031.CO5_HUMAN	Complement C5	Activation of C5 by a C5 convertase initiates the spontaneous assembly of the late complement components, C5-C9, into the membrane attack complex. C5b has a transient binding site for C6. The C5b-C6 complex is the foundation upon which the lytic complex i	0.04648260
sp.Q6UXB8.PI16_HUMAN	Peptidase inhibitor 16	May inhibit cardiomyocyte growth.	0.04648260
sp.Q9P121.NTRI_HUMAN	**Neurotrimin**	**Neural cell adhesion molecule.**	**0.04648260**

We performed string analysis to check the THD-severity network and we obtain 27 nodes, some of them highly connected. Regarding functional enrichments in the network we found the following categories enriched:

Table 23: Functional enrichments' categories in the network THD-severity

Category	Subcategory	False discovery rate (FDR)
GO (Biological process)	Cell Adhesion	0,00013
GO (Cellular component)	Chylomicron	7,72E-05
KEGG Pathways	Cell Adhesion Molecules (CAMs)	0,0005
Reactome Pathways	Neurofascin interactions	0,00083

Figure 22: String analysis of network THD-severity

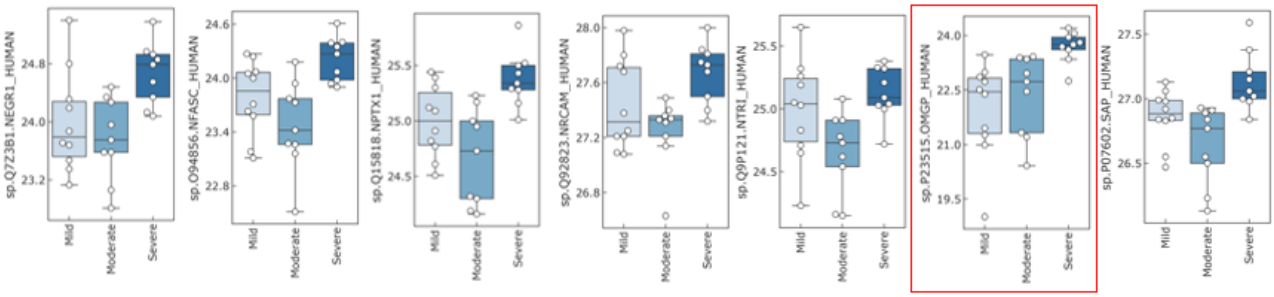


We plotted a selection of those 27 significative proteins in box plot graphs to observe how these proteins increase or decrease according to severity in THD patients. We split them in two groups: brain-related proteins or others.

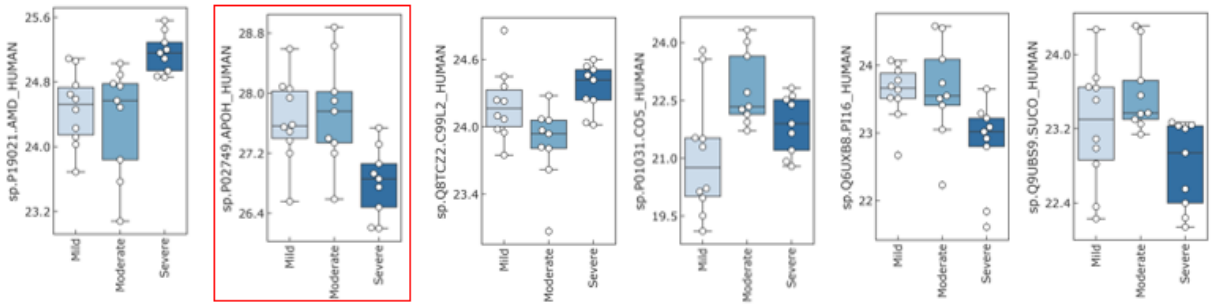
We selected for further analysis the brain-related protein Oligodendrocyte-myelin glycoprotein (OMGP) as it is important in myelination in the brain and we found increased levels in more severe patients. As a not brain-related protein we chose Apolipoprotein H or Beta-2-glycoprotein 1 (Apo H), which binds to various kinds of negatively charged substances such as heparin, phospholipids, and dextran sulfate. Apo H decreases with severity.

Figure 23: Box plots of variables THD-severity

A) Brain related proteins



B) Other proteins



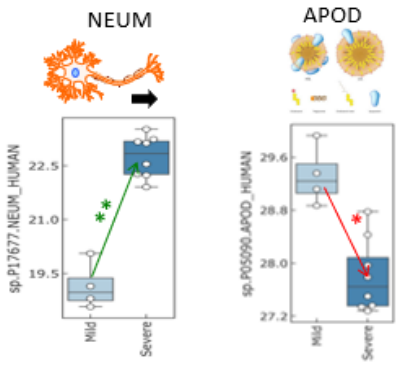
2.5.6 Biomarkers validated

After the proteomic study was carried out and the following analysis were performed, we wanted to validate the selected proteins with a second protein detection technique (such as ELISA) to check the trend described by mass spectrometry and assess if they could be good candidates for biomarkers in the different groups of disorders in our studied population.

2.5.6.1 AADC deficiency

In the case of AADC deficient patients we selected, as stated before; NEUM as a brain-related protein and APOD for the ELISA validation.

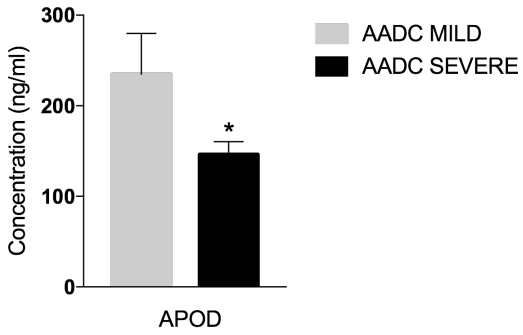
Figure 24: Boxplots of the proteomics study (AADC-severity)



For the ELISA validations it was possible to study a total of 6 AADC patients in the case of NEUM (as we needed at least 200 µl) but it was not possible to detect this protein in the CSF with the available ELISA kit (low detection range: 0.156 ng/ml).

We assessed the expression of APOD in 17 patients as we had to dilute the samples 1/5 to quantify them. In this case we observed how severe patients presented lower levels of APOD when compared to mild patients (p-value <0.05), confirming and validating the proteomics results.

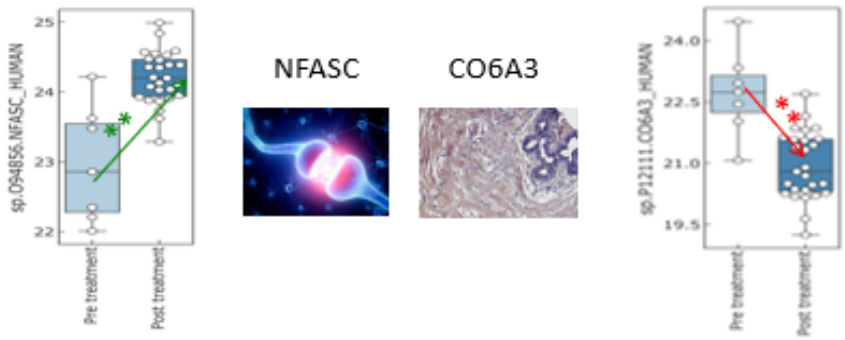
Figure 25: Graph representing the expression of APOD in AADC patients



2.5.6.2 BH4 disorders

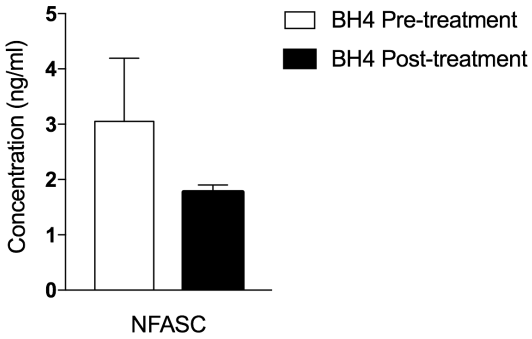
In the case of the BH4 disorders group we selected NFASC and COL6A3 for further ELISA validation:

Figure 26: Boxplots of the proteomics study (BH4 group-treatment)



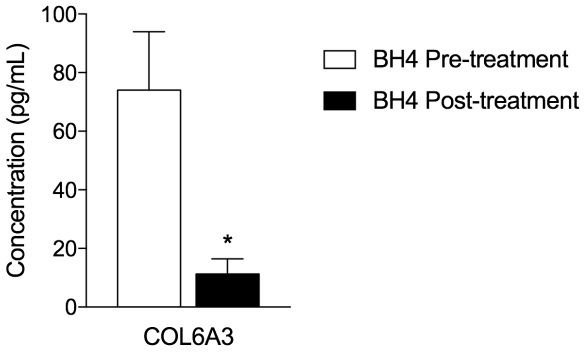
For the ELISA validation of NFASC it was possible to study a total of 27 BH4 patients. Even it was possible to detect this protein in the CSF with the corresponding ELISA kit; we observed a trend where NFASC expression decreased in posttreatment samples (the contrary that it was observed in the proteomics study), so it failed validation.

Figure 27: Graph representing the expression of NFASC in AADC patients



We assessed also the expression of COL6A3 in 16 patients without any dilution. We observed how the expression of COL6A3 decreased after treatment in a significant manner (p-value <0.05), then confirming the proteomics results and validating COL6A3 as a possible biomarker.

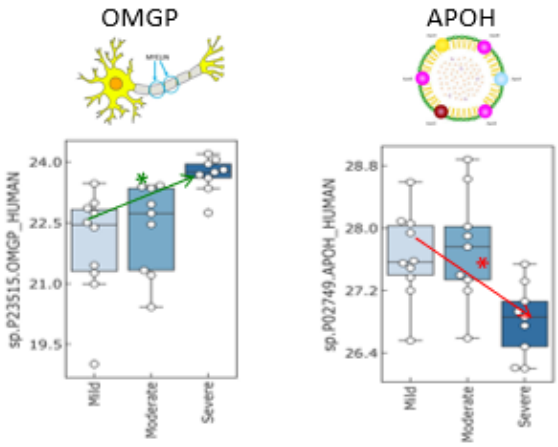
Figure 28: Graph representing the expression of COL6A3 in BH4 patients



2.5.6.3 TH deficiency

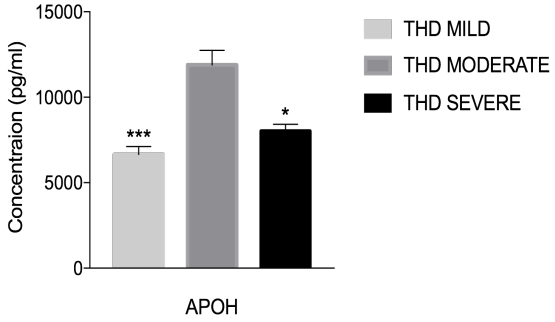
Finally, we assessed how was the expression of OMGP and APOH in THD patients depending on the patients' severity and compared it with the proteomics results.

Figure 29: Boxplots of the proteomics study (THD-severity)



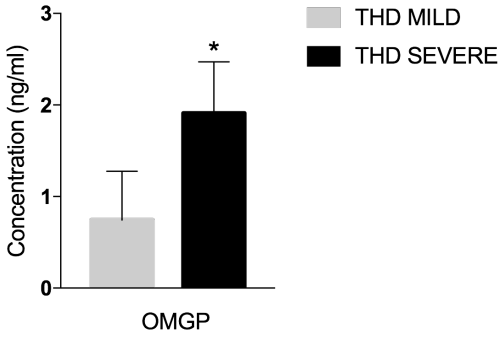
We assessed the expression of APOH in 28 THD patients and we were able to detect its expression diluting 1/5 the CSF sample. We observed an altered expression of APOH regarding severity which was significant but it didn't follow the same trend that was obtained with the proteomics study.

Figure 30: Graph representing the expression of APOH in THD patients



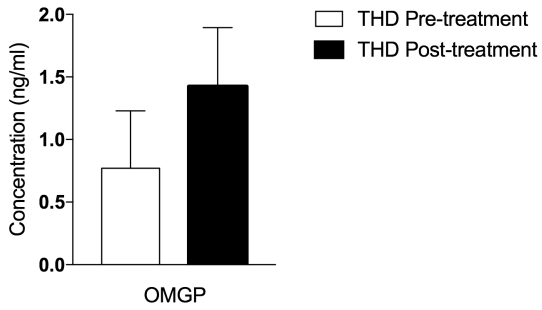
In the case of OMGP, its expression was studied in 25 THD patients and we were again able to detect the protein in the non-diluted CSF sample. We observed an increased expression of OMGP expression in severe patients when compared to mild ones. This result would confirm the previous proteomics results (p-value <0.05).

Figure 31: Graph representing the expression of OMGP in THD patients



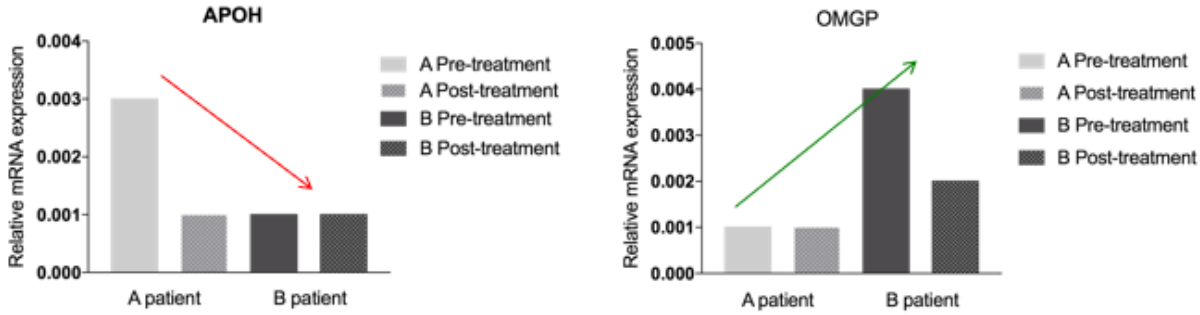
Moreover, we wanted to check in THD patients how OMGP was distributed when treatment was assessed. We graphed the previous assessed patients classifying them in pre and post-treatment samples. As it was observed in the boxplot of disease-treatment, in THD patients OMGP expression increases with treatment, even if it's not significant.

Figure 32: Graph representing the expression of OMGP in THD patients regarding treatment



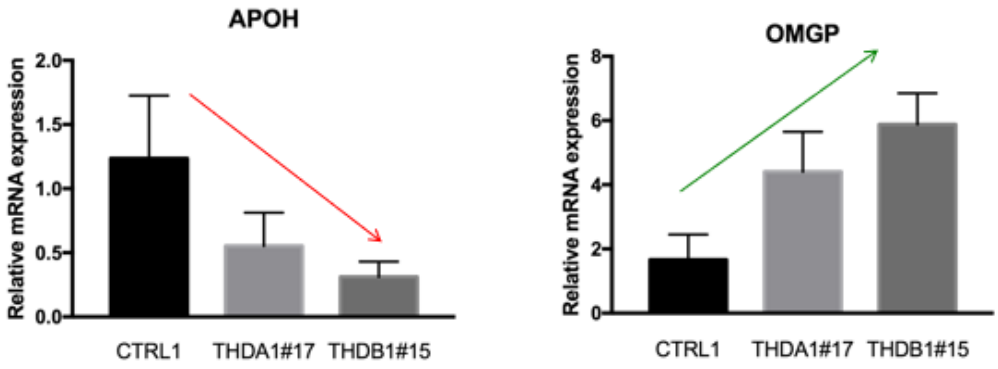
Taking profit of our cell model, we think it would be interesting to check how OMGP and APOH RNA expression was in our mild patient (THDA1) vs severe patient (THDB1) and how it respond to the treatment (cells treated with L-dopa + carbidopa).

Figure 33: Graph representing RNA expression of APOH and OMGP in our cells (untreated or treated with L-Dopa + carbidopa)



In the case of APOH expression we observed a reduced expression in APOH in the B patient when compared to the A patient but no clear effect regarding the treatment. OMGP expression is increased in B patient when compared to the A patient but no differences were found when pre an post-treatment samples were assessed. Even though, this experiment has been done just once.

Figure 34: Graph representing RNA expression of APOH and OMGP in our cells (mild vs severe)



We observed a reduced APOH expression in B patient when compared to the A patient and the control. OMGP expression is increased in B patient when compared to the A patient and the control. Although no signification was found (n=3), we confirm the trend that was observed in the patients CSF (both proteomic and ELISA results).

As a final summary of the proteomics results:

- This is **the first proteomic study** in a representative population with monoamine defects.

- The descriptive analysis shows that our population present the **same biochemical alterations** described in the literature for each of the disorders studied.

- The enrichment analysis shows that the **main category of overrepresented proteins** was related to nervous system development.

- Different proteins were detected that could be useful **biomarkers for severity prognosis and response to treatment** that are specific of disorders. After a more detailed analysis **6 were chosen for their validation** for a second protein method detection: **APOD** and **NEUM** for AADC; **NFASC** and **COL6A3** for BH4 disorders and **APOH** and **OMGP** for TH deficiency.

- From these 6 proteins, **4 were correctly validated with an ELISA analysis**: **APOD** (AADC), **COL6A3** (BH4 disorders) and **APOH** and **OMGP** for THD patients (also validated with our THD iPSCs based model).

APOD is involved in the transport of diverse compunds; **COL6A3** has an structural function as cell binding, **APOH** binds to phospholipids and other molecules and **OMGP** is important in myelination processes in brain.

- More studies were needed in the future to confirm its feasibility as possible **biomarkers** and/or **therapeutic targets**.

3. Cell model based on iPSCs

3.1 Generation and characterization of iPSCs

We performed a skin biopsy punch to isolate fibroblasts from two controls and two patients (one with A phenotype and another with B phenotype) to generate iPSCs clones with a non-integrative strategy (episomal vector) . Bellow the clinical information of the patients and controls:

Table 24: Clinical information of the patients and controls

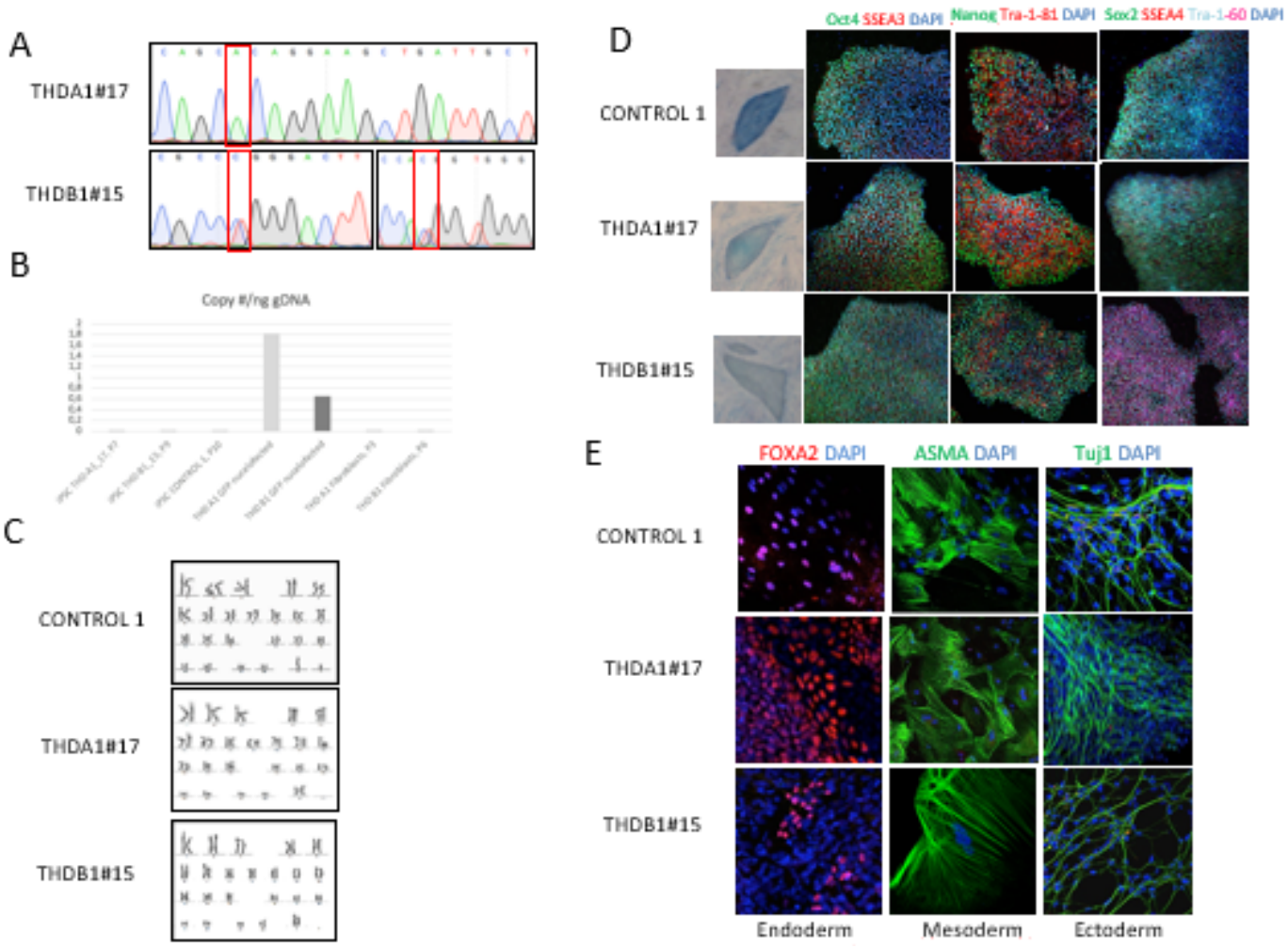
	PATIENT				DISEASE							
	Code	Sex	Age at biopsy punch (years)	Origin	Onset age	Age at diagnosis/ treatment	Symptoms	Cognitive Function	Phenotype	Response to L-Dopa	BQ markers (nmol/l)	Mutations
CONTROLS	CONTROL 1	M	9,00	Spanish	-	-	-	-	-	-	-	-
	CONTROL 2	M	3,00	Spanish	-	-	-	-	-	-	-	-
PATIENTS	PATIENT 1	F	9,00	Spanish	4m	11m/11m	Tremor (++), hypokinesia (++), rigidity (+)	Normal, IQ (tested at 4.5 years)	A	Good	HVA: 158 (range: 344-906) HVA/HIAA: 0.64 (range: 1.5-3.5)	p.R233H /p.R233H
	PATIENT 2	F	16,00	Spanish	5m	3y/3y	Oculogyric crisis (++), tremor (+), hypokinesia (++), rigidity (++), autonomic dysfunction (+)	Severe mental retardation, No language	B	Slow improvement. Autonomous gait. Initially dyskinesias after slow L-Dopa increases	HVA: 15 (range: 304-658) HVA/HIAA: 0.05 (range: 1.5-3.5)	p.R328W /p.T399M

The following tests were carried out to fully characterize the iPSCs lines generated from our individuals: first a mutational screening in the iPSCs was performed to check if they had the same mutation found in the patients.

All cell lines were positive for the AP staining indicating their fetal origin and we confirm the absence of the episomal vector. All the cell lines have normal karyotype, the controls are males and the patients are female.

Apart from that, the cell lines are positive for the different pluripotency markers (Oct4, SSEA3, Nanog, Tra-1-81, Sox2, SSEA4 and Tra-1-60). And they were also able to generate the 3 germ layers, as we can see positive staining for the endoderm (FOXA2), mesoderm (ASMA) and ectoderm (Tuj1) markers.

Figure 35: Characterization of a selection of these iPSCs lines



A: Chromatogram representing the sequence of the TH gene of the iPSCs lines of study B: Graphic showing the copy #/ng of gDNA. C: Karyotype study of the iPSCs D: Alkaline phosphatase (AP) staining and immunocytochemistry studies with pluripotent markers. E: Immunocytochemistry studies with three germ layers' markers.

We generated iPSCs lines from controls and patients, with at least two clones characterized for each patient (bellow the information of the characterization tests performed).

An isogenic control was also obtained editing the line THDA1#17 with CRISPR/CAS9 technology.

Table 25: Summary of iPSCs lines generated

	PATIENT					CHARACTERIZATION					
	Code	Sex	Age at biopsy punch (years)	Origin	Mutations	Clones	Karyotype	AP staining	Episomal vector	Pluriptency markers	In vitro diff.
CONTROLS	CTRL1	M	9	Spanish	-	CTRL1#5	46, XY	+	absence	OK	OK
	CTRL2	M	3	Spanish	-	CTRL2#8	46, XY	+	absence	OK	OK
ISOGENIC CONTROL					p.R233H /p.R233H corrected	THDA1#17 corrected	46, XX	-	absence	-	-
PATIENTS	THDA1	F	9	Spanish	p.R233H /p.R233H	THDA1#5	46, XX	+	absence	-	-
						THDA1#17	46, XX	+	absence	OK	OK
	THDB1	F	16	Spanish	p.R328W /p.T399M	THDB1#1	46,XX	+	absence	-	-
						THDB1#15	46,XX	+	absence	OK	OK

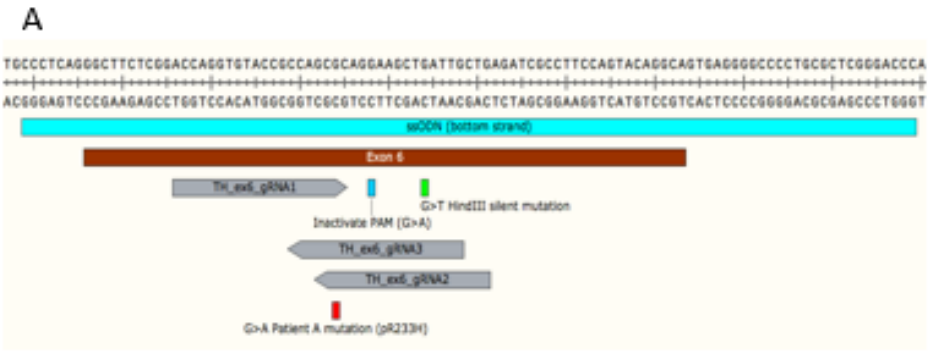
3.1.1 Gene correction of THDA1#17 iPSCs line

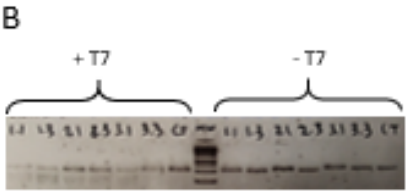
Regarding the gene correction of the THDA1#17, we selected the region of the exon 6 where the mutation R233H locates and designed three different guide RNAs and a DNA template (ssODN) that had the mutation corrected, inactivate the PAM sequence to avoid repeating cuts and a new restriction enzyme site for HindIII to facilitate the positive confirmation of the gene edition.

To choose the best guide RNA an endonuclease T7 assay was performed and after checking the % of cleavages we chose gRNA 1 (average of % of gene modification was 35%).

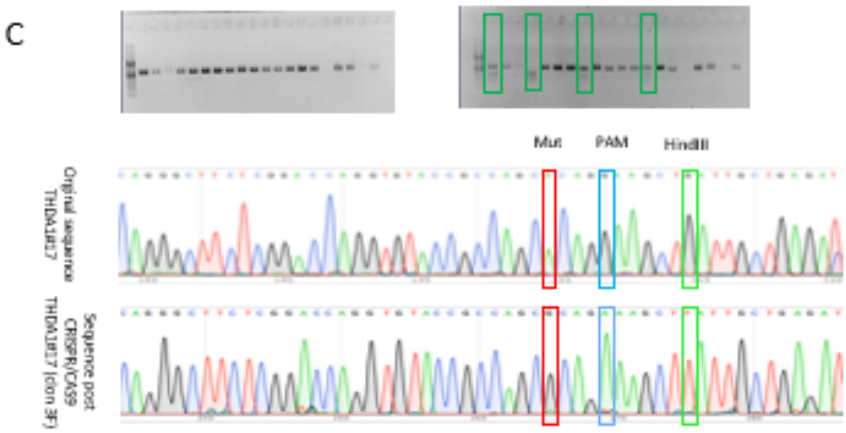
After the nucleofection of our clone of iPSCs, we move forward to the selection of positive clones. We first performed a PCR of this part of the exon 6 that was digested with HindIII to check the possible positive clones and later on sequenced to confirm the changes in the chromatogram. We found 13 clones positive for the digestion and 4 after the sequence study. We selected clones (3F1H and 3A3A) for doing some passages to guarantee monoclonality and confirm also the karyotype stability. After its confirmation, we selected the clone 3F1H for the following studies.

Figure 36: Gene editing strategy and results of the IPSCs line THDA1#17





		Area	Gene mod. (%)
gRNA1.1	Uncut	2.060.234	28,56
	Cut	1.976.569	
	Sum	4.036.803	
gRNA1.3	Uncut	2.181.891	42,08
	Cut	4.322.175	
	Sum	6.504.066	
gRNA2.1	Uncut	5.463.962	13,71
	Cut	1.873.790	
	Sum	7.337.752	

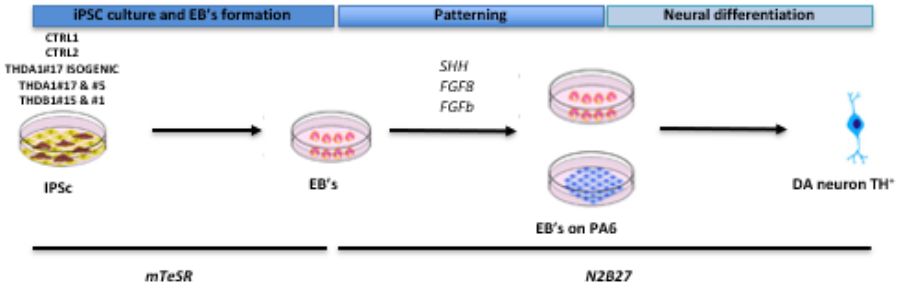


A: Region of the exon 6 with the ssODN, the gRNA and the positions for PAM sequence, the mutation correction and the new restriction site for HindIII B: T7 endonuclease assay and calculation of gene modification (%) C: PCR of exon 6, digestion of this PCR product with HindIII and sequence of a positive clone (3F) where the mutation is corrected, the sequence PAM is inactivated and the a new restriction site it's introduced; all of them In homozygosis D: Normal karyotype is observed for the clone 3F1H

3.2 Generation and characterization of Dopaminergic neurons

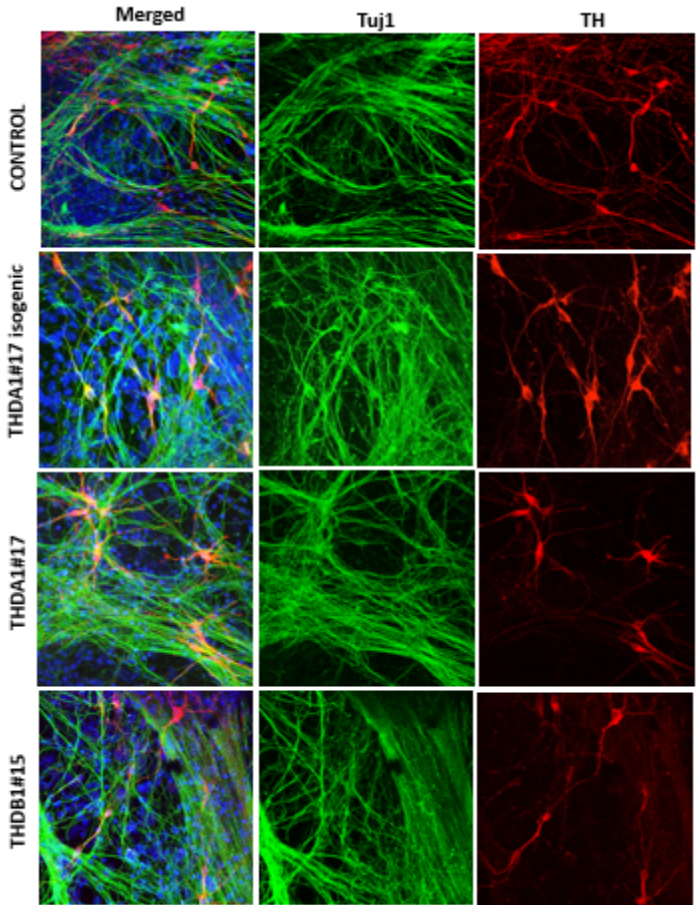
Dopaminergic neurons were generated from all these iPSCs lines after a 3w differentiation protocol based on EBs generation and dopaminergic patterning factors.

Figure 37: DAn neurons for all these lines were generated following a 3w protocol



After these 3 weeks of differentiation immunochemistry studies were performed to confirm that we had dopaminergic neurons expressing TH+ marker and neurons in general (Tuj1) in all the cell lines. After this confirmation, we could continue with the studies on dopaminergic neurons.

Figure 38: Representative image of the obtention of Dopaminergic neurons



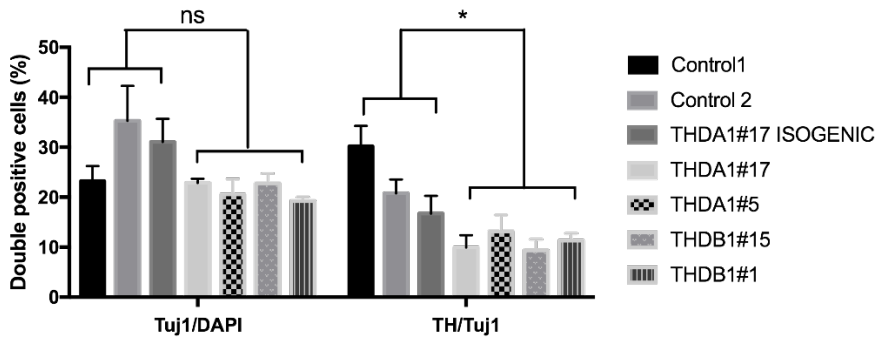
The markers TH in red as a marker of Dan, Tuj1 in green as a marker of general neurons and DAPI that stains the nucleus.

3.3 Mutant TH+ neurons show less TH immunoreactivity

To assess if all the cell lines presented the same number of neurons and dopaminergic neurons we quantified the ratio Tuj1/DAPI and TH/Tuj1.

When we took into account the ratio TH/Tuj1 we have quantified less TH+ neurons in the DAn derived from THD patients compared to control cultures (p-value <0.05), even though we obtain the same number of neurons (no differences are observed regarding the ratio Tuj1/DAPI).

Figure 39: Graph representing the ratios TUJ1/Dapi and TH/Tuj1

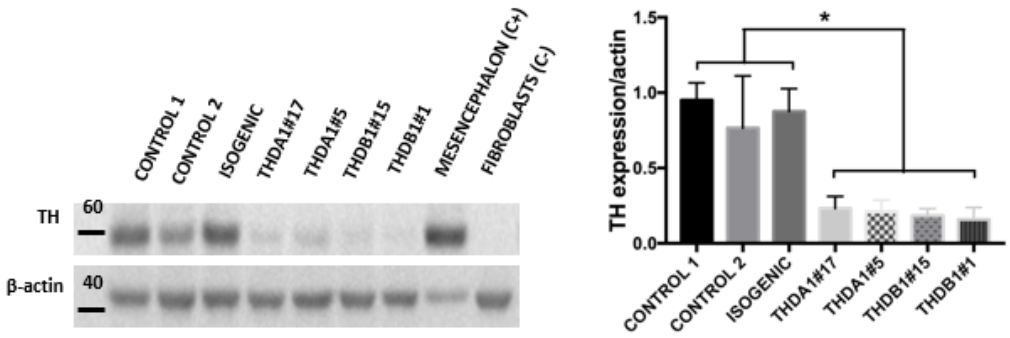


3.4 THD neurons display a reduced amount of TH protein and TH activity

We then assessed the expression of the TH protein by WB in all the cell lines (including the isogenic and two clones of each patient), compared to a positive control (mesencephalon) and a negative control (fibroblasts); all corrected by actin.

We observed a reduction in TH protein in the patient derived DAn compared to the control ones when we corrected by actin. In fact, it seems that both clones for THDB1 iPSCs line present lower levels than A1 iPSCs line, even if it doesn't reach significance.

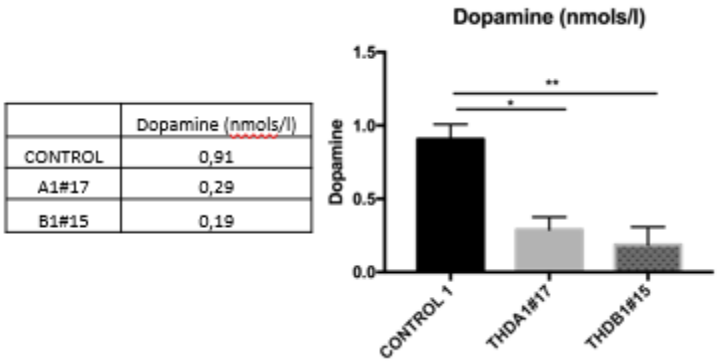
Figure 40: Western blot bands and quantification of TH expression



To study how the enzyme TH is working we assessed Dopamine levels inside the cell and in the supernatant together with DOPAC (Dopamine metabolite).

For doing so we performed first an ELISA analysis to quantify the quantity of intracellular dopamine in the control and in a clone of each patient. We could already observe how intracellular dopamine is reduced in both patients in comparison with the control, which is more evident in the B patient clone than in A clone (p-values <0.01 and <0.05 respectively).

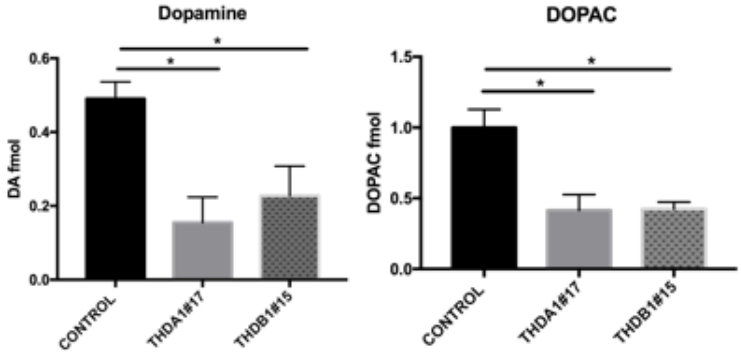
Figure 41: Intracellular dopamine quantification with an ELISA



	Dopamine (nmols/l)
CONTROL	0,91
A1#17	0,29
B1#15	0,19

We have also performed HPLC studies to assess the levels of dopamine and DOPAC in the supernatant. We were able to see that the DAn derived from THD patients have also lower levels of Dopamine and DOPAC when compared to control (p-value <0.05)

Figure 42: Quantification of Dopamine and DOPAC levels in the cell supernatant with HPLC



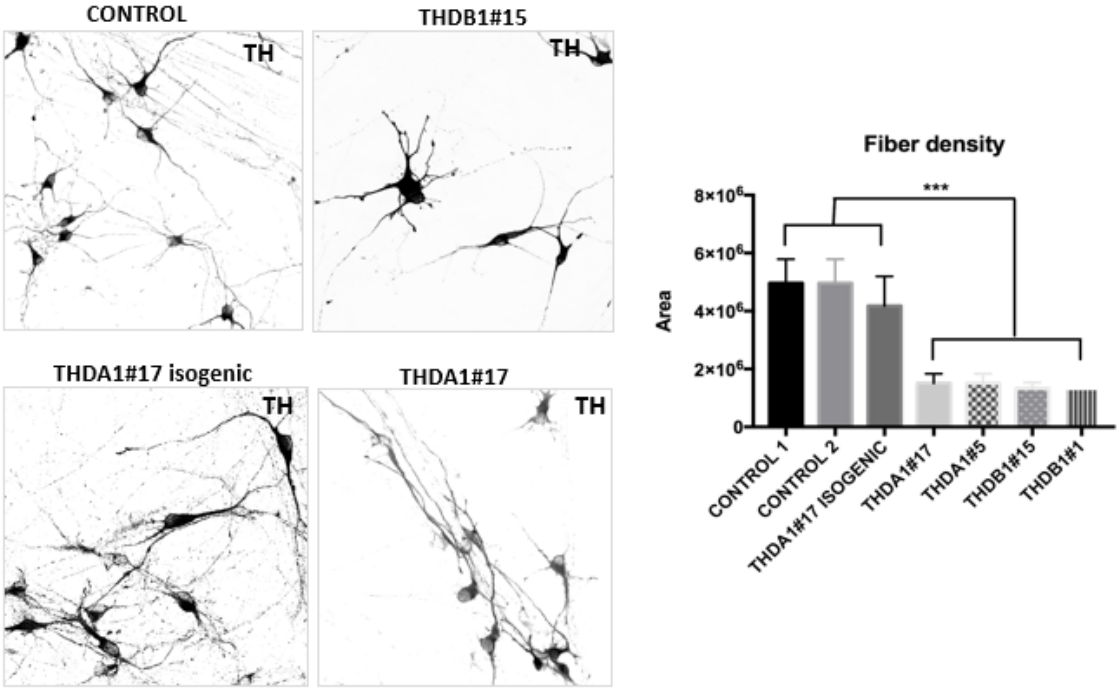
3.5 Mutant TH+ neurons show less fiber density

We have also observed that the cultures of DAn derived from THD patients had less neurites or arborisation stained with TH.

For measuring this we took into account the area of the field that was occupied with TH axons and neurites but discarding the somas.

We observed that in both THD cultures we had less area occupied with this fibers (less fiber density).

Figure 43: Images and quantification of fiber density

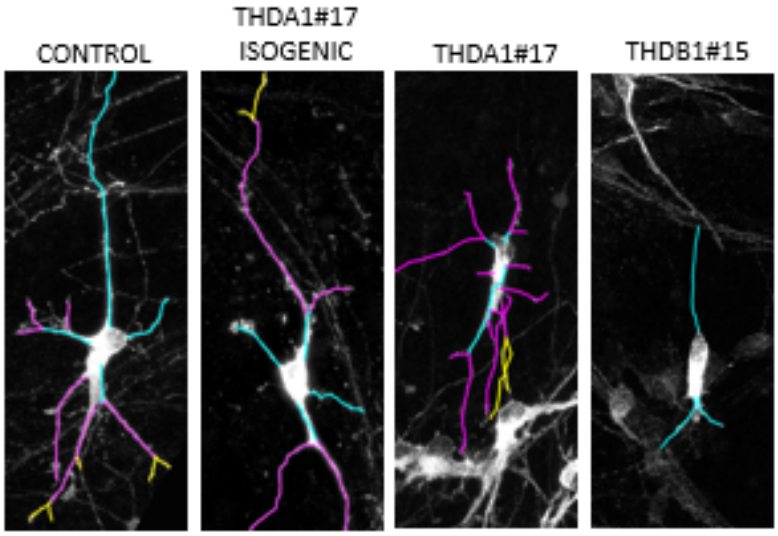


3.6 Both patient's TH+ neurons show altered morphology

As we could already observe with the images it seemed that THD patient's TH + neurons had a different morphology when compared to controls.

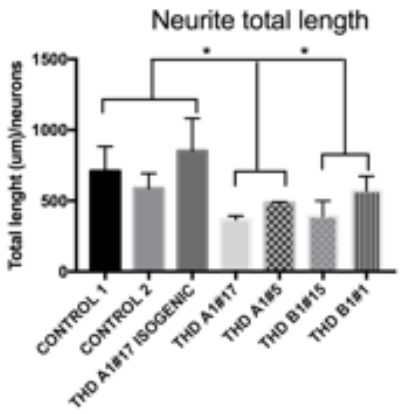
To quantify this difference, we performed a tracings study where different colours are used to label or draw the neurites (primary neurites are labelled with blue, secondary ones with purple and tertiary neurites with yellow).

Figure 44: Images of the tracings



When we analyse the tracings, we observed that the neurite length was reduced in THD patient TH+ neurons, to quantify this, we took into account the neurite total length (which is he sum of the length of all the neurites in a TH+ neuron). We could observe a reduction in this neurite total length in both THD A and THD B TH+ neurons when compared to controls, which is significant (p-value <0.05).

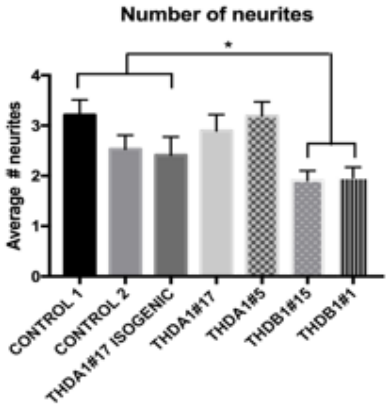
Figure 45: Graphic quantifying the neurite total length



3.4 THD-Type B neurons show reduced neuronal arborisation

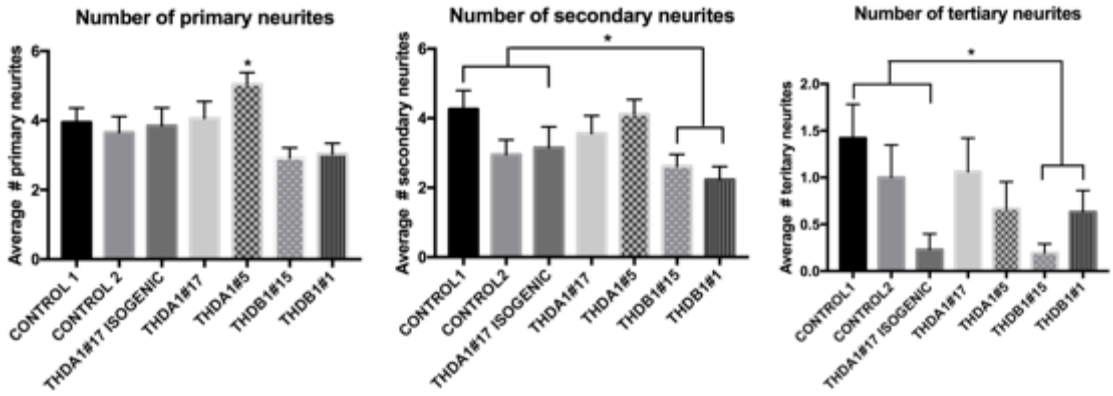
Apart from the affected neurite length, we observed that the B patient TH+ neurons presented also a reduced number of neurites, so we quantify this and we obtain a significant difference (p-value <0.05) when comparing both clones of B patients with the controls and the isogenic lines.

Figure 46: Graphic quantifying the number of neurites



To further address which type of neurons were affected we quantify the number of primary, secondary and tertiary neurites. We observed a reduced number of primary neurites (even not significant) and a reduction of secondary neurites and even more clear fewer tertiary neurites in both B clones when compared to controls and isogenic lines (p-value <0.05).

Figure 47: Graphic quantifying the number of the different type of neurites



3.7 THD-Type A neurons show reduced axonal TH localization

Comparing the soma with the last distance point in the axon were the growth cone locates, we already observed an increase in the ratio soma/last point in the axon in the DAn derived from A patient (both

clones) when compared to control ones and isogenic (p-value <0.01) that was not observed in B patient's clones.

Figure 48: Images of TH intensity in some of the lines of study

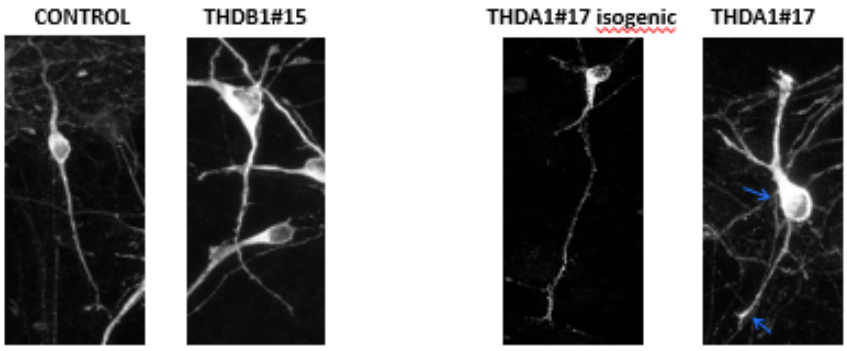
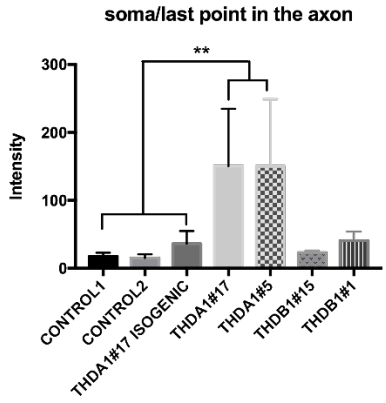
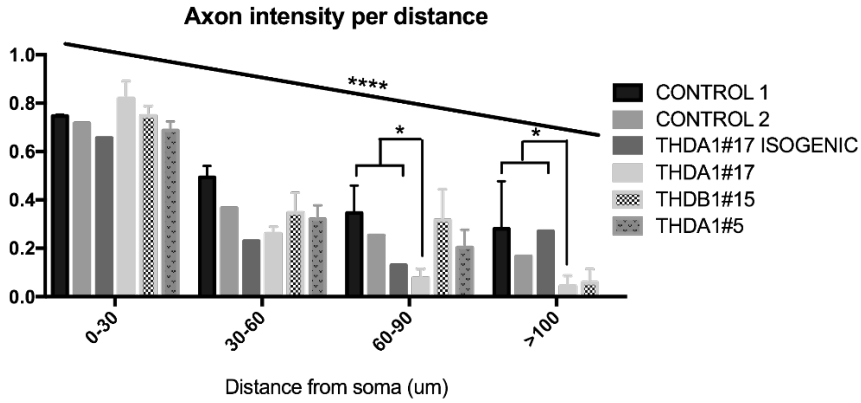


Figure 49: Graph representing the differences in TH intensity between soma/last point in the axon



Also, when we assessed how is TH intensity per distance, we observed lower intensities along the axon that is more pronounced in the A patient DAn (p-value <0.05) suggesting a possible defect in TH transport.

Figure 50: Graph representing the differences in TH intensity along the axon



3.8 Dopaminergic genes expression

Then we thought that it would be interesting to assess how different dopaminergic genes are expressed in our cell model.

For doing so we extracted RNA from one control and one clone of each patient after 3 weeks of differentiation.

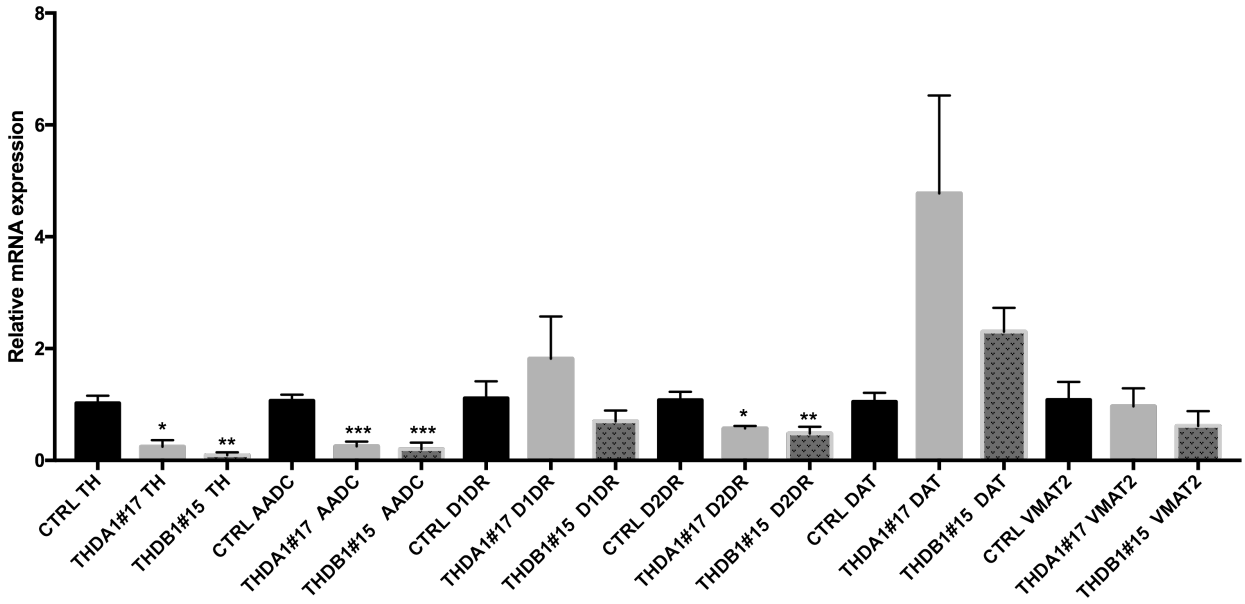
We observed a reduction of TH expression in both patients derived neurons (that is more pronounced in B cell line that in the A (p-value< 0.01 and p-value <0.05, respectively).

There was also a reduction in AADC expression which is highly significant in both A and B lines (p-value <0.001) when compared to the control.

Again, we observed significant differences in the D2DR expression that is more pronounced in B phenotype than in A phenotype (p-value <0.01 and p-value <0.05, respectively).

We also observed some expression differences in D1DR, DAT and VMAT2 but it didn't reach statistical significance.

Figure 51: Graph representing the expression of DA genes



3.9 Treatment assessment

3.9.1 Chaperone treatment

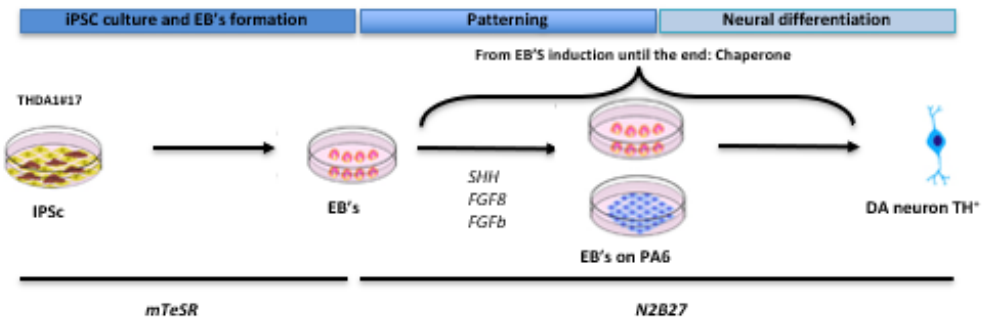
We tested a chaperone that had promising results in a PC12 cell model previously generated in our laboratory (Hole et al. 2015) to assess if it could somehow restore and recuperate TH activity or transport among other features in our cell model.

We choose the compound II which chemical formula is "*(2E)-3-(2-chlorophenyl)-N-{4-[(3,4-dimethyl-1,2-oxazol-5-yl)sulfamoyl]phenyl}prop-2-enamide"* now on named chaperone 2 (Chap. 2).

We performed different trials using different concentrations dose of this peptide. We decided to test first a dose of 100 µM on one control and the two phenotypes' iPSCs lines and we observed a lethal effect on the EBs (disgregation) in both conditions but more pronounced in the chaperone treatment, so we finished the treatment and sacrified the cells.

On a second trial, we test two different concentrations of the chaperone 2 in the THDA1#17 iPSCs line. The first dose of 50 µM resulted again in cell death, now only in the chaperone-treated cells.

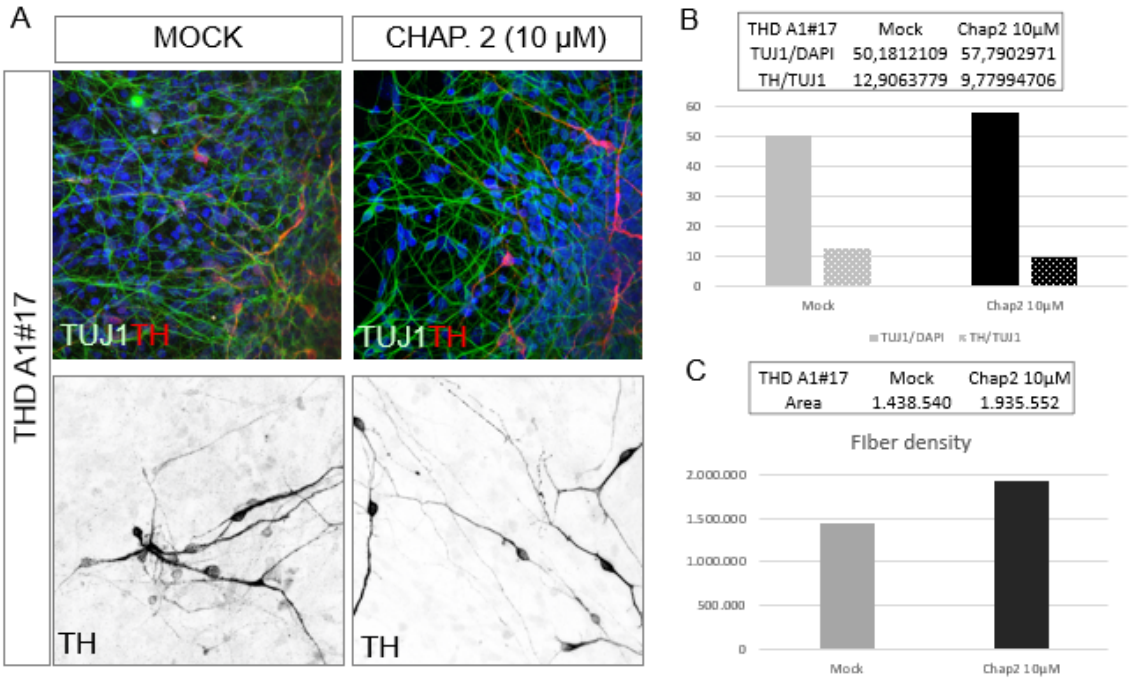
Figure 52: Chaperone treatment was given to the THDA1#17 line during all the process



With the second dose of 10 µM the cells survived and therefore were treated during all the differentiation protocol (n=1).

Regarding the results obtained with the 10 µM vs mock, no big differences were observed in the treated vs untreated culture regarding the counting of Tuj1/DAPI or TH/Tuj1 ratios. Fiber density was also quite similar between the two conditions.

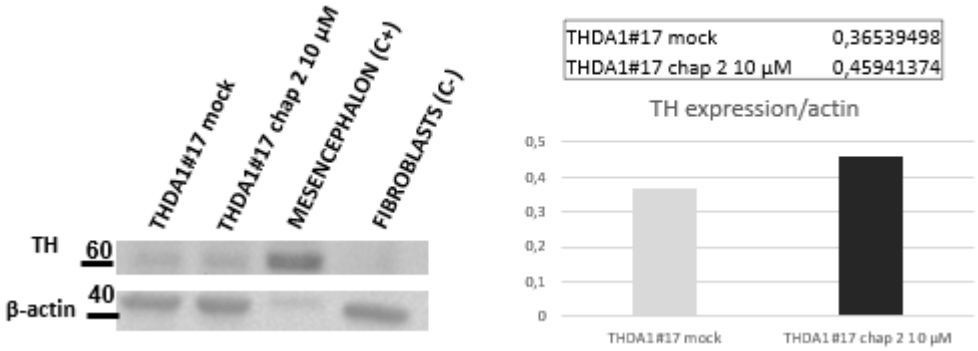
Figure 53: Images and analysis of the cultures treated with chaperone 2



A: Immunochemistry studies with the staining of TH in red and Tuj1 in green, below the image in black and white to address fiber density. B: quantification and graph of the Tuj1/DAPI and TH/ Tuj1 ratios. C: quantification and graph of the mean area of TH intensity or fiber density.

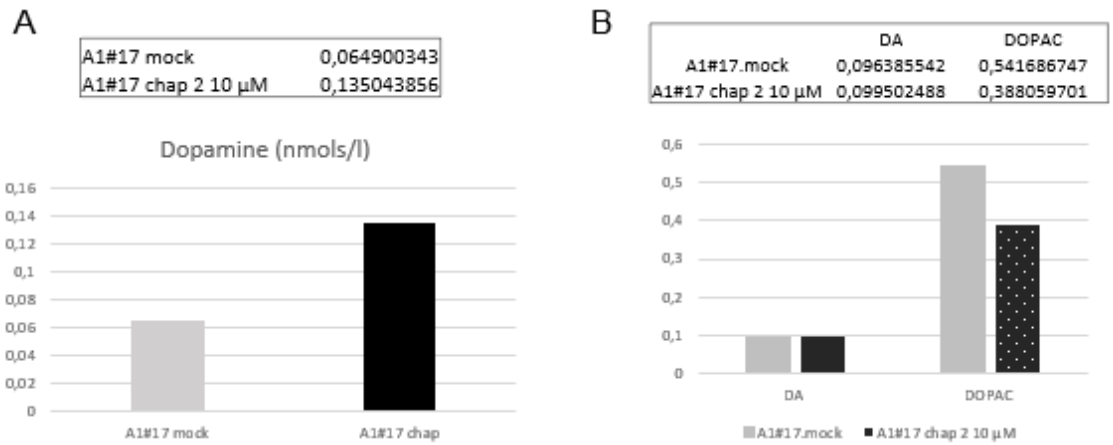
When we studied how the chaperone treatment affected the TH expression by Western Blot, we didn't observe any differences between mock and treated THDA1#17 cultures (again with a positive and a negative control):

Figure 54: Western Blot studies in the cultures treated with chaperone 2



Finally, to assess TH activity issues we measured the intracellular dopamine by ELISA and the levels of Dopamine and DOPAC in the cell supernatant with HPLC technique. In both cases we didn't find any clear difference between treated and untreated cultures.

Figure 55: TH activity addressed by ELISA and HPLC studies



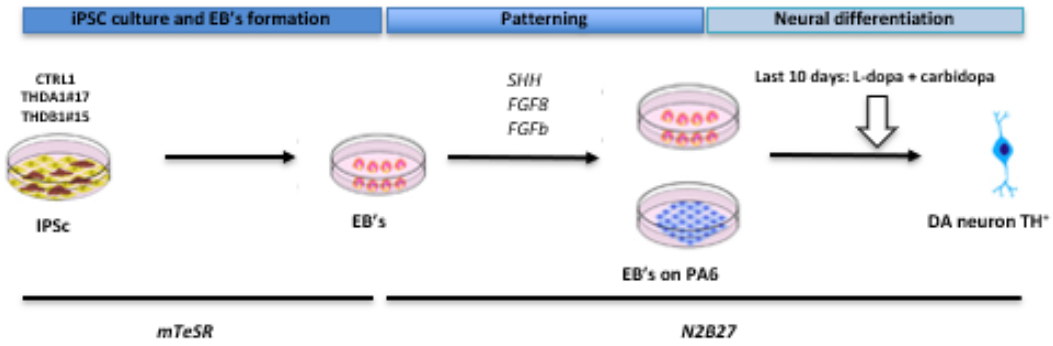
A: Quantification and graph of the intracellular dopamine levels measured by ELISA B: HPLC quantification of dopamine and DOPAC levels in the supernatant.

3.9.2 L-Dopa + carbidopa treatment

We also wanted to test the usual treatment that is given to THD patients, so we treated our cells with a combination of L-Dopa (50 µM) and carbidopa (12.5 µM) in a ratio of 1:4.

In this case we treated the DAn derived from one control and the two phenotype patients

Fig 56: Treatment of the following iPSCs lines with L-Dopa + carbidopa



Regarding how the cell cultures respond to the treatment of L-Dopa + carbidopa (stated only as L-dopa in advance), we could observe how the control and the A phenotype patient derived DAn had an increase in both the number of neurons in general and TH+ neurons (as judged by the ratios Tuj1/DAPI and TH/Tuj1).
In the case of B patient cultures we could only observe an slightly increase in TH/Tuj1 ratio after the L-dopa treatment.

Figure 57: Images of the cultures treated with L-Dopa + carbidopa

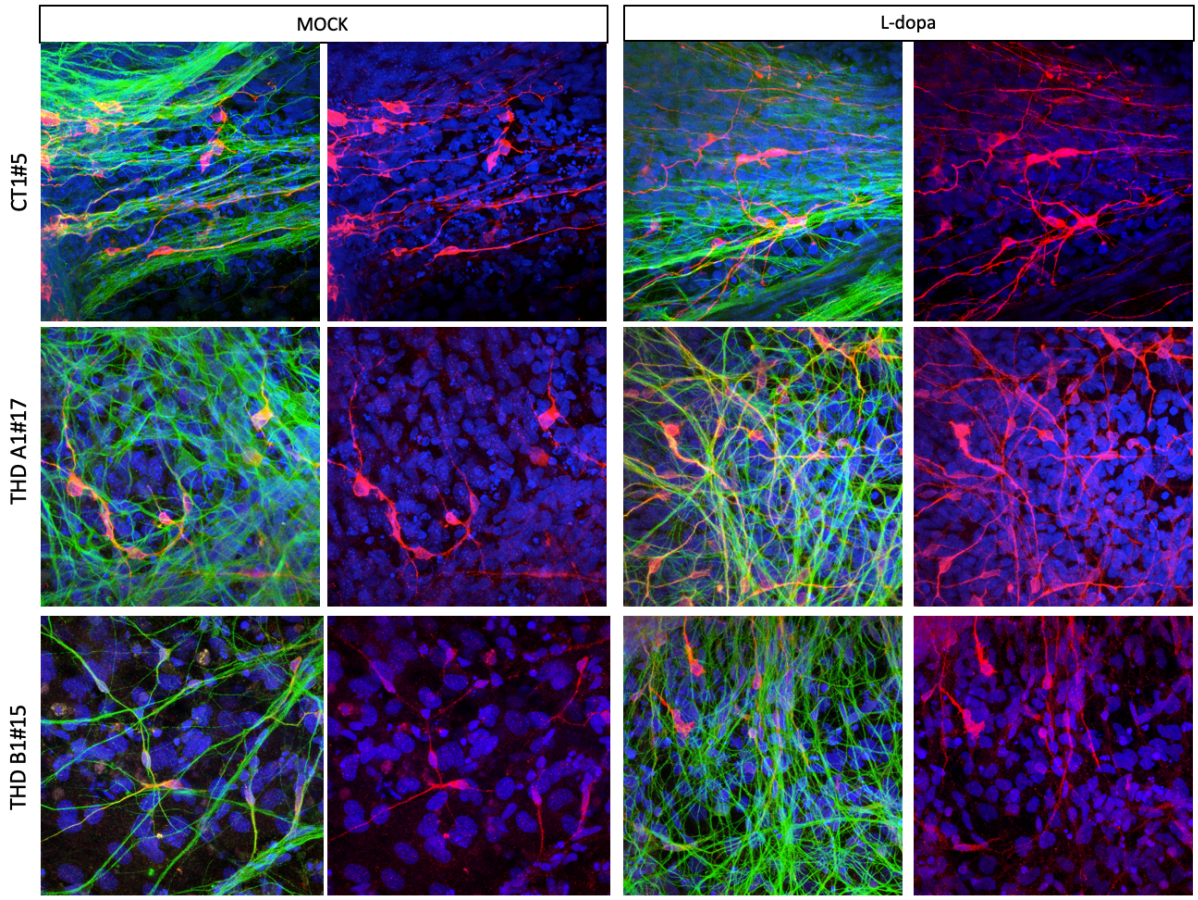
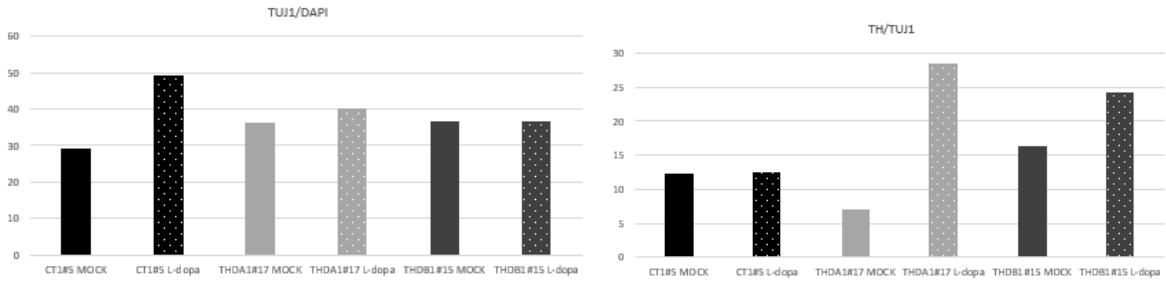


Figure 58: Graph representing the ratios TUJ1/Dapi and TH/Tuj1 after L-dopa treatment



Then we analysed the area that was occupied by TH axons and dendrites (fiber density) and we observed an increase in fiber density in both the control and THDA1 patient cultures after L-dopa administration that was confirmed by its quantification.
It was not possible to observe this effect on the B patient derived- DAn after the treatment.

Figure 59: Images for fiber density analysis in the cultures treated with L-dopa

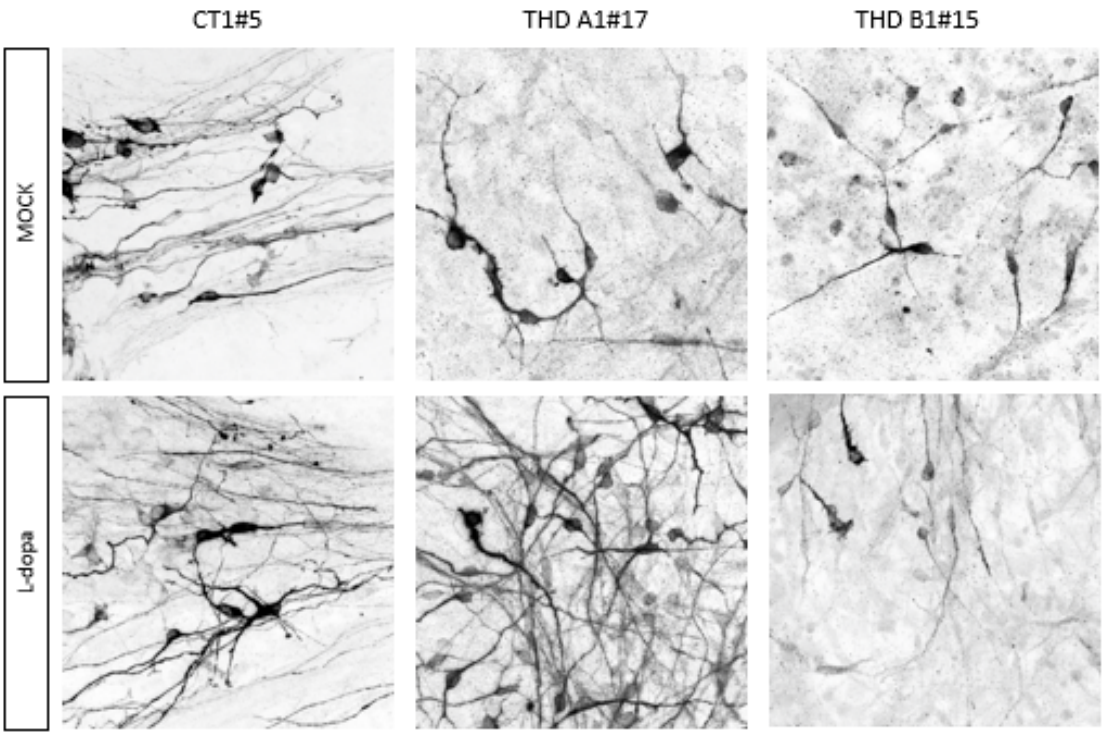
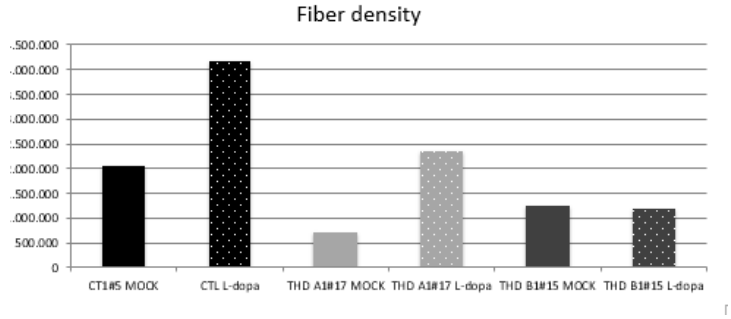


Figure 60: Quantification of fiber density analysis in the cultures after L-dopa treatment

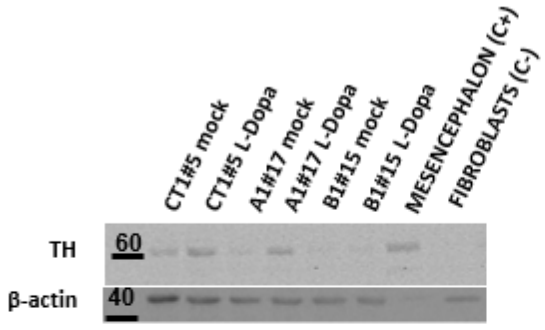


We performed western blot studies to assess how TH expression was affected with the treatment of L-dopa + carbidopa.

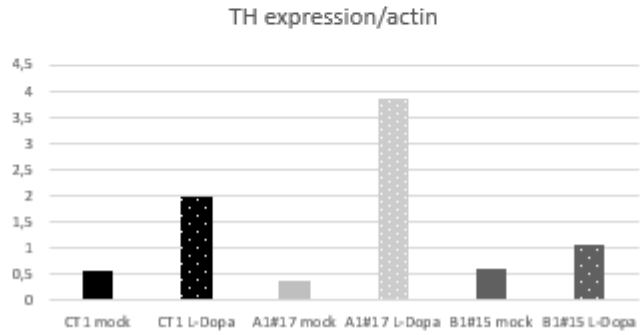
We observed how in all the cases the expression of TH increased after the treatment even though it was more evident in the control and THDA1#17 when we quantify the optic density of the bands and corrected by actin (no statistical analysis was performed as is n=1).

All was done with a positive and a negative control for TH protein expression

Figure 61: Western Blot studies in the cultures treated with L-Dopa



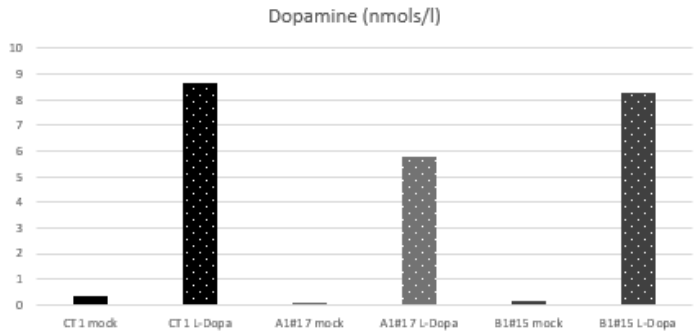
	TH/ACTIN
CT1 mock	0,562527379
CT1 L-Dopa	1,975757198
A1#17 mock	0,372190878
A1#17 L-Dopa	3,850845659
B1#15 mock	0,612273446
B1#15 L-Dopa	1,063190561



To study how L-dopa treatment affected to the intracellular levels of Dopamine we measured this dopamine with an ELISA assay. We observed how with the intracellular DA levels were increased after the treatment in all the cell lines, with no major differences among them.

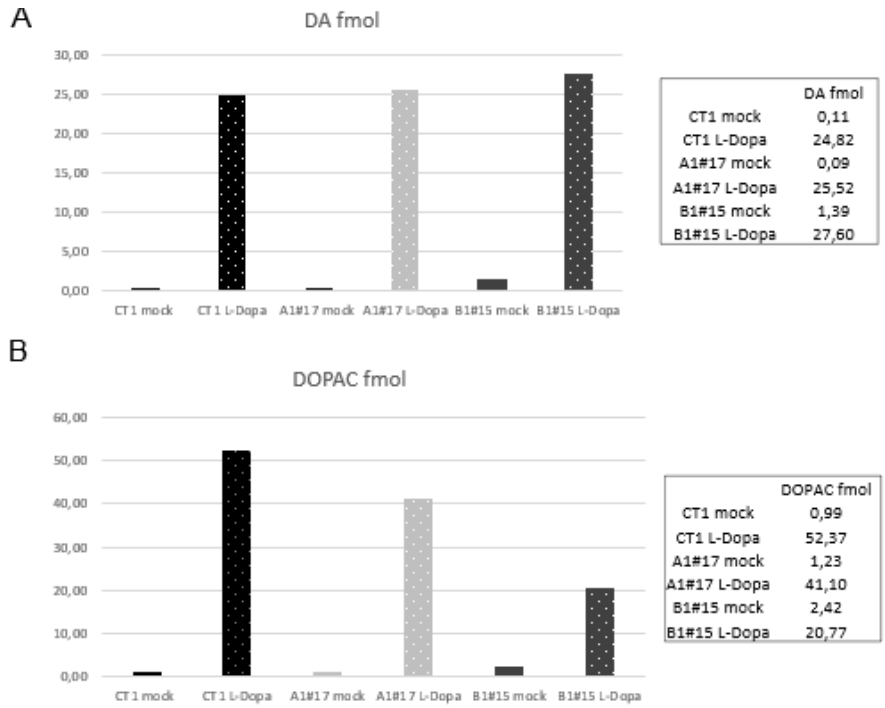
Figure 62: Levels of intracellular dopamine assessed by ELISA

CT1 mock	0,34068641
CT1 L-dopa	8,65310686
A1#17 mock	0,08923355
A1#17 L-dopa	5,78411095
B1#15 mock	0,15816097
B1#15 L-dopa	8,24432467



Finally, we measured the levels of Dopamine and DOPAC in the cell supernatant with HPLC technique. In both cases we found an increase of both metabolites when compared untreated and treated samples with no differences between lines.

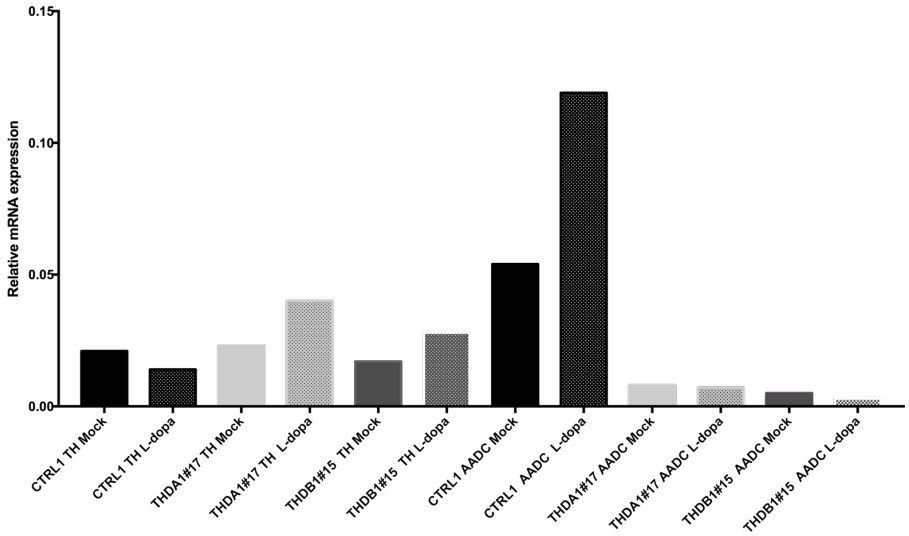
Figure 63: Levels of extracellular dopamine and DOPAC assessed by HPLC



A: Quantification of the levels of DA in the supernatant; B: Quantification of the levels of DOPAC in the supernatant

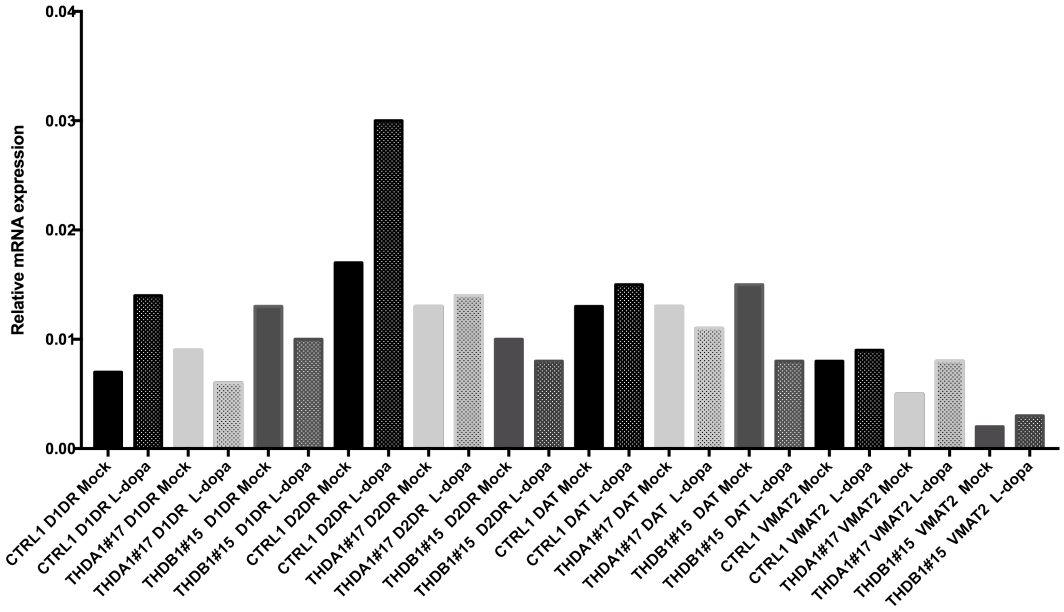
Finally, we wanted to assess how L-dopa treatment affected to different DA genes expression:
In the case of Dopaminergic enzymes we observed and increase in the RNA expression of TH in both patients but not in the control and an increase of AADC in the control but not effect was observed in the patients derived DAn.

Figure 64: DA enzymes gene expression



In the case of other DA genes expression we observed an increase in D1DR and D2DR expression in the control derived DAn after the treatment that was not observed in the DAn from the patients. The same profile was observed in the case of the dopaminergic transporter DAT. Regarding the VMAT2 we observed an increase in all the cell lines after the L-Dopa treatment.

Figure 65: Other DA genes expression



As a final summary of the iPSCs results:

- A new THD model based on iPSCs technology has been established
- The new model recapitulates the **disease phenotype,** such as the reduced TH expression and activity, due to the mutation. We also observed alterations in the expression of different DA genes.
- A new **neuronal phenotype** has been identified:
    - less TH-immunoreactive cells
    - less fiber density in both mutant TH+ neurons
    - altered morphology (Type A and B)
    - reduced neuronal arborisation observed in THD-Type B
    - reduced axonal TH localization observed in THD-Type A
- Regarding the **treatments** tested in our cell model:
    - This specific dose of the chaperone 2 was not able to produce any effect in our cell cultures.
    - The treatment of L-Dopa + carbidopa produce the full recovery of both the disease and neuronal phenotypes in the A phenotype DAn neurons. Some of them were also restored in B phenotype derived cultures but in a slightly manner.

DISCUSSION

THD postmortem brain

Since DA is one of the first neurotransmitters released in the brain, it's likely to play a crucial role in neurodevelopment, regulating neurogenesis, migration, spinogenesis and synaptogenesis.

Therefore, a deficiency in DA synthesis and thus in the modulation of all these processes, can lead to alterations in brain connectivity (Money and Stanwood 2013). Mouse models show that alterations in genes involved in catecholamine synthesis result in a global dopaminergic dysfunction and impaired fetal survival (Kobayashi et al. 1995; Q.-Y. Zhou and Palmiter 1995). It has also been described that dopaminergic neurons need all of this enzymatic and transport machinery for the correct synthesis, storage and release of DA (Prakash and Wurst 2006).
In our study, we have measured the expression of key neuronal proteins to assess how different neurotransmission pathways and neurodevelopmental biomarkers are affected.

We observed an under-expression in almost all of the dopaminergic proteins in our patient. The under-expression of VMAT 1 and 2 and the alterations found in AADC expression, may indicate changes that could worsen a deficiency in dopamine that's already deleterious. This is plausible considering the global maturation and functioning that biochemical pathways undergo at early stages of brain development. Hence the lack of TH as a key enzyme in dopamine synthesis, can affect the expression and regulation of other enzymes, transporters and receptors involved in the dopaminergic pathway (Prakash and Wurst 2006).

D1DR and D2DR involved in the basal nuclei direct and indirect pathways (Zeiss 2005), were also under-expressed in the patient. In particular D2DR was under-expressed, suggesting an imbalance between both pathways that could be related to the signs of parkinsonism that appear later Iravani et al. 2012). Additionally, D2DR modulates neuronal morphology in the frontal cortex and striatum, which could have further harmful consequences in learning and behaviour functions (Money and Stanwood 2013).

The changes observed in glutamatergic and GABAergic markers suggest that DA is an important regulator of other neurotransmitters and could support the GABA-dopamine co-release hypothesis (Zeiss 2005; Tristsch et al. 2012). Therefore, the decreased GABAVT expression in our patient may reflect a reduced GABAergic function consistent with low DA content. Changes in NMDAR1 expression could be related to DA regulation through its binding to D1DR and D2DR (Tsui and Isacson 2011).

The decreased expression of PSD95 may suggest a decreased number of synaptic connections. The under expression of NF-H and MAP2 implies a reduction in axons and dendritic density. In contrast, the neuronal volume seems to be unchanged when compared to the control due to the similar expression of NSE. These findings point towards the idea of THD as a neurodevelopmental disorder involving not only dopaminergic deficiency but also issues with synaptic and brain maturation.

Other techniques such as immunohistochemistry and RNA microarrays could validate and assess other aspects of this study, however on arrival the entire sample was processed to extract proteins and unfortunately further research could not be performed.

Proteomics study

For this study, we include the following patients with diverse neurotransmitter defects: 29 TH, 17 AADC, 9 GTPCH, 8 DHPR, 22 PTPS, 4 SR and one DAT with ages from 0 to 30 years (average age of 6 years). Of these patients, 39 were males and 51 females.
Some of these patients have been given treatment and others not and they present a highly variable clinical symptomatology with mild, moderate or severe phenotypes.
All along we think this is a large and representative population with monoamine defects.

Regarding the descriptive studies in our population we confirmed the biochemical values' alterations described in the bibliography (Ng et al. 2015) as we observed the following features in our population that is in line what has been described in this previous article and others:

Table 26: Biochemical features in our population in accordance with previous studies

NT defect/ Biochemical marker	AADC	SRD	PTPS	GTPCH	DHPR	THD
HVA	↓	↓ or =				↓
5-HIAA	↓	↓ or =				↓ or =
HVA/ 5-HIAA		↓ or =				↓
3-OMD	↑					
NP		↑ or =	↑	↓ or =	↑ or =	
BP		↑ or =	↓	↑ or =	=	

In our population, we observed that all THD patients present low levels of HVA and that these values are even lower in the most severe patients (p-value 0.09).

Also, we could observe that after the L-Dopa + carbidopa treatment severe patients continue to have low or unchanged levels of HVA; whereas mild and moderate patients are able to increase or normalize those HVA levels.
As expected, a similar effect is observed when taken into account the ratio HVA/5-HIAA.
All together, these results reinforce HVA as a good biomarker for the diagnose and prognosis of THD (Willemsen et al. 2010).

Analysing the results of the proteomic studies we could observe that the main category of the 385 common detected proteins was related with nervous system development. In fact, the enrichment studies performed confirmed that those proteins were related to neurodevelopment processes such as: plasticity, excitability and synapse maturation among others.

We could therefore state that this NTD of monoamines are neurodevelopmental disorders and that there should be a change in the paradigm on how these disorders are addressed in the clinics and research fields and redefine them (Tristán-Noguero and García-Cazorla 2018).

We have found several proteins that could be useful biomarkers for severity and prognosis specific for diseases:

ApoD (AADC)

For AADC deficiency it was possible to validate the protein ApoD, where low levels of this protein correlate with a more severe phenotype in the patients.

Apolipoprotein D is a lipoprotein that is not able to form a lipoprotein particle itself, but rather associates exposing their hydrophobic residues with other lipoproteins or HDL particles and several lipids such as cholesterol, steroids or arachidonic acid.
ApoD is expressed in several regions of the brain like cortex and cerebellum. Brain ApoD is synthesized and excreted predominantly by neurons and glial cells (Elliott, Weickert, and Garner 2010).

It is known that ApoD binds and modulates arachidonic acid triggering an anti-inflammatory and antioxidant effect. This could explain why the overexpression of ApoD in mouse or Drosophila confers resistance to oxidative stress increasing the survival and life span respectively (Dassati, Waldner, and Schweigreiter 2014).

Endogenous ApoD increases during aging process and is altered in some psychiatric and neurological

diseases:

An increase in the ApoD CSF levels has been described in patients with meningoencephalitis, stroke, dementia and motor neuron disease. ApoD is also increased in the dorsolateral prefrontal cortex in schizophrenic patients and in a mouse model of Niemann Pick (Elliott, Weickert, and Garner 2010).

High levels of ApoD have been also described in Parkinson's disease: in glial cells of postmortem human brains of PD patients and validated later in human plasma of PD patients at the late stage of the disorder (when neurodegeneration and astrogliosis processes have already started) when compared to controls (Waldner et al. 2018).

One the other hand; Apo D is decreased in blood samples in patients enduring a sepsis process where they described other alterations in lipids homeostasis and other proteins related to energy metabolism (Sharma et al. 2017). It is also decreased in Alzheimer disease; as it would have a neuroprotective role against neuronal cell death and in disease, they link low levels of ApoD in the CSF of AD patients to a defective lipid transport and reduced repair processes prompting to neuronal degeneration (Oláh et al. 2015).

In our population of AADC patients we observed that severe patients presented lower levels of ApoD when compared to mild patients (p-value <0.05) and this would go in the same direction of what is observed in other disorders where neurodegenerative processes are not a hallmark (in those cases an increase in ApoD levels is described).

ApoD reduced levels in the most severe AADC patients go in line of a failure in the control of these ROS stress processes and anti-inflammatory effect which will also produce an accumulation of peroxidated lipids (impaired neuronal homeostasis). ApoD has also been described as a lipid transporter protein in neurite-promoting responses, in active myelination and in axonal outgrowth as well as a regeneration-promoting agent, so if this fails it could impair important functions needed for the correct development of these patients' brain.

Moreover, treatment interactions shouldn't be ruled out as L-Dopa or monoamine oxidase inhibitors could exert a potential down regulation of ApoD due to their antioxidant effects. This effect could be stronger in severe patients as they don't usually respond well to treatment and sometimes higher doses are given (Waldner et al. 2018).

Regarding mouse models where they knock out the gene ApoD, they observed a peroxidation of brain lipids, decreased life span and diverse motor and cognitive defects. The loss of ApoD disrupts proteostasis machinery and induce oxidative and inflammatory damages (Bhatia et al. 2018).

ApoD has already been proved as a reliable biomarker for the diagnosis and progression of some disorders such as Parkinson's disease among others so it could be also useful to measure it in the CSF of AADC patients. Also taking into account that it is also expressed in human plasma we could try to revalidate this marker in AADC plasma samples which could help us avoid invasive procedures such as the lumbar puncture.

Regarding their utility as a possible therapeutic target according to Li et al. 2017; upregulating the levels of ApoD (pharmacologically or by other means) could slow down the neurodegeneration process given in PD through their binding and transporting molecules that could produce neurotoxic effects on dopaminergic neurons and becoming a promising treatment for PD patients.
All in all, make ApoD a promising therapeutic target for a huge range of different neurologic diseases and aging ameliorating and/or delaying its progression through its anti-stress and anti-inflammatory activity. During the next years it would be interesting to investigate methods to upregulate its endogenous expression levels or find ways to delivery it exogenously to the CNS (Dassati, Waldner, and Schweigreiter 2014).

COL6A3 (BH4 disorders)

COL6A3 was validated by ELISA in our BH4 disorders group as we observed a decreased expression in posttreatment samples.

Collagen VI is an extracellular matrix protein expressed ubiquitously. It assembles into dimers and tetramers to finally form microfibrils with a structural function of a wide variety of tissues, including skeletal muscle, skin, and cartilage (Pan et al. 2013).
Even though, according to Domingo, Erro, and Lohmann 2016; collagens are not limited only to structural adhesion function, they also have important roles in the formation and maintenance of neural circuits.

COL6A3 have been involved in different disorders:

It has been described as a potential prognosis marker for colorectal cancer (increased in cancer samples) in a quantitative proteomics study (Qiao et al. 2015).

Moreover, mutations in the gene COL6A3 (together with COL6A1 and COL6A2) encoding different collagen chains can produce Ulrich congenital muscular dystrophy and Bethlem myopathy or phenotypes between them (Pan et al. 2013).

Demir et al, 2002 also describe that mutations in COL6A3 cause severe and mild phenotypes of Ullrich Congenital Muscular Dystrophy.

Compound heterozygous mutations have been also described producing isolated dystonia, which is characterized by involuntary twisting postures arising from sustained muscle contractions (without muscular disease). All affected individuals had at least one pathogenic allele in exon 41 (Zech et al. 2015).
Interestingly, higher expression of COL6A3 mRNA was detected in the dentate gyrus of Alzheimer's disease patients compared with controls.
Moreover, the treatment of murine primary neuron cultures with the neurotoxic cleavage product of APP stimulate the expression of COL6. Which in turns, prevent the interaction of this neurotoxic product with neurons and thus protecting them. This neuroprotective effect is not exclusive of APP as a similar effect has been observed in UV- induced apoptosis (Cheng et al. 2009).

Exist some zebrafish models where they knockdown the COL6A3. They observe a defective axonal targeting, a kind of phenotype that is also observed in neurodevelopmental disorders so they hypothesized that COL6A3 mutations disrupt the establishment of normal neural circuits during development (Domingo, Erro, and Lohmann 2016).
Zech et al. 2015 studied in more detail the involvement of COL6A3 exon 41 in pathology, for doing so, they induce a series of in-frame deletions in zebrafish exon 41 ortholog, and they observe a defect in axonal outgrowth.
They were also able to demonstrate its expression in adult brain mouse neurons by RNA expression analyses.

All things considered will indicate that the loss of function mutation in COL6A3 can produce different muscular dystrophies, myopathies or isolate dystonia which also underlies a neurodevelopmental deficit in some cases.

COL6A3 has only been described as a biomarker in colorectal cancer and increased in AD to confer a neuroprotective effect but in our case, we have found that its expression decreases with the treatment in BH4 disorders patients.
It would be interesting to confirm the feasibility of using this biomarker for treatment assessment in an unrelated population. Also check if we could detect COL6A3 in peripheral tissue samples (such as blood or plasma) to avoid using so limited and unreachable samples as cerebrospinal fluid.

Regarding its possible therapeutic use; according to Gregorio et al. 2018; COL6A3 has been shown to be neuroprotective under stress conditions, as well as in neurodegeneration. In fact, is hypothesized that COL6A3 might affect the correct early development of neuron circuits, as well as the synaptic remodelling of adult brain. This is in agreement with the finding that another ECM protein, tenascin-C, is able to modulate synaptic plasticity and axonal outgrowth thus suggesting a role for the α3(VI) C4 domain in synaptogenesis and in the maintenance and/or stability of neural circuits.

APOH (TH deficiency)

We validated the biomarker ApoH in TH deficient patients as we saw alterations in protein levels by ELISA analysis and a decreased level in severe patients confirmed by lower RNA expression levels in DAn neurons derived from B patient in comparison with A patient in our THD cell model.

Apolipoprotein H is a lipoprotein that binds the complement and coagulant factors such as XI and thrombin and also regulates angiogenesis and interact with the atherosclerotic plaque. β2GPI (alternative name for ApoH) it has also a role in the innate immune response, in fact, during the acute phase of an infective inflammation ApoH levels decreased.
β2GPI is also able to interact with apolipoprotein E receptor 2 (APOER2), a member of the low-density lipoprotein (LDL). A role in early embryonic development in mice has also been observed (El-Assaad, Krilis, and Giannakopoulos 2016).
It circulates freely but about a 35% is associated with lipoproteins and binds to negatively charged substances, including heparin, phospholipids, and dextran sulfate (Song et al. 2012).

Apolipoprotein H has been found altered in different disorders:

The antiphospholipid syndrome (APS) is an autoimmune disease characterised by a procoagulant state that predisposes to recurrent thrombosis and miscarriages. APS could lead also to neurological complications including vascular brain disease and increased risk of developing dementia. It was described that beta-2 glycoprotein-1 (β2GPI) or ApoH is the major auto antigen in APS (El-Assaad, Krilis, and Giannakopoulos 2016).
ApoH may prevent activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells. High levels of pathogenic ApoH antibody in turn could cause hypercoagulation and venous and arterial thrombosis, and is clinically relevant to APS (George and Erkan 2009).

Increased levels of ApoH are found in the CSF of patients with dementia (subcortical vascular dementia (SVD) and mixed dementias (MD)) and moderate-severe Alzheimer Disease patients when compared to controls (Öhrfelt et al. 2011).

Also increased levels of ApoH were found in the CSF of PD patients when using shotgun proteomic quantitative strategies (Licker and Burkhard 2014).

(Heywood et al. 2015) performed a label-free proteomic study and also found the biomarker apolipoprotein H significatively elevated in the CSF of neurodegenerative conditions such as Alzheimer's Disease, Lewy Body Dementia and Parkinson's disease (PD) when compared to controls.

In another study by (Song et al. 2012) they show low levels of Apolipoprotein H in the plasma of mild cognitive impaired patients when compared to controls. They also described how ApoH low levels increase the risk of cognitive decline over two years in cognitively normal individuals. In fact, ApoH levels are positively correlated with global cognitive scores, attention/processing speed and executive functions.

Regarding mouse models: it has been described an interaction between high ApoH antibody levels and the load of amyloid plaques in an AD mouse model (APP695SWE mutation mice immunized with ApoH) (Katzav et al. 2011).
In a mouse model of systemic lupus, the deletion of ApoH produced an increase of autoantibodies levels, inflammatory cytokines and the presence of lymphadenopathy and splenomegaly. In a mouse model of cardiac IRI, damage is reduced by treatment with the fifth domain of β2GPI as its inhibits the inflammatory cascade (El-Assaad, Krilis, and Giannakopoulos 2016).

Apo H has been proved to be a good biomarker in plasma and CSF samples of diverse neurological patients with disorders such as APS, dementia, AD or PD.

It would also have a link with cognitive decline which could explain relatively well the low levels we found in the most severe patients of our THD population. THD severe patients (B phenotype) usually present with developmental delay, mental retardation and autonomic dysfunction and poor response to L-dopa so they have worse cognitive prognosis than mild patients (A phenotype).

Moreover, it would possible to be found in plasma samples and not only in CSF.

Regarding its therapeutic potential; even this protein has not been clearly linked on how affects brain and is involved in neurological disorders and a cognitive decline; it could be hypothesized that

subclinical hypercoagulation caused by low ApoH levels might increase the risk for this symptomatology (Song et al. 2012). According to this, it would be really interesting to increase somehow the endogenous levels of ApoD or give it exogenously to confer a protective effect.

OMGP (TH deficiency)

We validated the protein OMGP as a biomarker in our THD population. We observed an increased expression in severe patients when compared to mild ones (p-value<0.05).
It was also possible to evaluate OMGP expression at the RNA level in our THD cell model and we observed the same trend of higher values in DAn derived from B phenotype patient (severe) when compared to the ones derived from A patient (mild) and the control.

Regarding the OMGP distribution after treatment we observed that OMGP increases in posttreatment samples even if it doesn't reach significance. In the THD cell model no differences were found when pre- and posttreatment samples were assessed.

Oligodendrocyte myelin glycoprotein (OMGP) is a glycosylphosphatidylinositol-anchored protein expressed mainly by neurons in the CNS and oligodendrocytes (Gil et al. 2010).

This glycoprotein localizes in the adult CNS, specifically in the neocortex, cerebellum, brainstem hippocampus, and the spinal cord. In more detail, cortex OMGP is found in neurons, axons, dendrites, synaptosome fractions and axon varicosities.
During development, OMGP expression goes with myelination; increasing from birth to early adult until it decreases and reaches a stable expression level in the adult (Geoffroy and Zheng 2014).

Regarding physiological roles of OMGP; this protein is a negative component of myelin that inhibits growth cone and neurite outgrowth like Nogo and MAG (Hasegawa et al. 2004).

A role for cell proliferation has been described for OMGP, in fact this gene is localized in an intron of the Neurofibromatosis type I, an important tumour expression gene. Overexpression of NF1 increases growth inhibition, the same effect observed with the OMGP overexpression.

Finally, OMGP has been also implicated in synaptic plasticity. When OMGP is exogenously added attenuates Long-term potentiation (LTP) response in hippocampal slices but not Long-term depression (LTD) suggesting its role in activity-dependent synaptic strengths. Even if its present in both presynaptic and postsynaptic density fractions, it does not regulate the release of presynaptic

neurotransmitters but it does have a role in postsynaptic function controlling LTP and synaptic strength (Geoffroy and Zheng 2014).

In vitro studies have shown that OMGP affects the proliferation of mesencephalon rat neural stem cells by inhibiting their multiplication rate (Martin et al, 2009) and that the treatment of cell cultures by OMGP inhibits the neurite growth and axonal regeneration in NG108 and PC12 cells (Kottis et al. 2002).
Regarding mice models; it has been described that the OMGP-null mice are hypomyelinated due to the modulation of the cell differentiation and myelination of oligodendrocyte precursors but not its proliferation (Fujita et al. 2011).
According to a study by Song et al. 2004; OMGP could also regulate axonal target specification during the development of thalamo-cortical projections as in the OMGP knockout mice there is an invasion of ectopic areas (layer II and III) instead of the normal one (layer IV).

OMGP has been implicated or described in different disorders:

OMGP is down-regulated in Alzheimer's disease explained by the myelin degeneration, impaired myelin-axon interactions, and node of Ranvier dysfunction observed in AD pathogenesis (Zhang et al. 2018).

OMGP is also decreased in the CAPN5-vitreoretinopathy when compared to controls as assessed by a proteomic study using vitreous samples (Velez et al. 2019). In this same study, they detected ApoH as an important molecule of transport in the Acute phase response network and related also with complement cascade.

Another study based on functional genomic and proteomic analysis reveals the disruption of myelin-related genes in a mouse model of early life neglect, among them a down-regulation of OMGP which is significant (Bordner et al. 2011).

An increase in OMGP Protein is observed after hypoxic-ischemic brain damage in neonatal rats (Liu and Li 2012).

Finally, Martin et al. 2009, described a genetic association between autism and particular alleles of a microsatellite and a single nucleotide polymorphism (SNP) in the *OMG* gene locus; but it would lack a better understanding on how OMGP works during brain development to fully characterized the role of this glycoprotein in autism aetiology.

They also show a recent study where they found a reduction in OMGP mRNA expression in the hippocampus of rats after 19 weeks of chronic ethanol consumption (Okamoto et al. 2006) and hypothesize that a down-regulation of OMGP expression may help to compensate or reduce the neuronal loss caused by ethanol neurotoxicity in regions such as the hippocampus.

OMGP is altered in different disorders and could be a good biomarker in AD, so it makes feasible their use in Tyrosine Hydroxylase deficiency, as it is increased with severity and after treatment with L-Dopa + carbidopa. Otherwise it has to be studied in more detail and in an independent population to be sure.

Regarding the possible therapeutic utility of OMGP, a decrease in the endogenous levels of OMGP could be a way to improve the severity in THD patients. Liu and Li 2012, propose the use of androgen (dose of 30 mg) to reduce OMGP expression in the hypoxic-ischemic brain in rats. Which in addition results in the reduction of the inhibition of axon growth and therefore, promoting axonal regeneration and producing a neuroprotective role in brain.

IPSCs model:

We have generated the first THD cell model based on iPSCs.

The major advantage of iPSCs among others disease models is that you can obtain patient and disease specific stem cells.
The best features of these cells are that they are human, with the same genetic background of your patient and most interesting with the ability to obtain cells of the 3 germ layers (endoderm, mesoderm and ectoderm) so you can differentiate them to your truly cell of interest

We isolated fibroblasts from two THD patients one with a milder phenotype and that responds to L-Dopa (A phenotype) and another with a more severe phenotype and that doesn't respond well to the treatment (B phenotype).

In our case we were able to generate iPSCs from two controls and obtain also an isogenic control from the mild phenotype with a new tool as the Crispr-Cas9 (Hockemeyer and Jaenisch 2017) which allow us to have a control with the TH mutation corrected but with the same genetic background as our A phenotype patient.

With all these tools, we were able to observe a disease-related phenotype already described in other THD studies:

First, we observed a reduced TH protein expression by Western Blot studies in DAn derived from both patients when compared to controls and isogenic ones. This was already described in the postmortem brain study (Tristán-Noguero et al. 2016) in a patient with B phenotype. Also in a PC12 cell model (Hole et al. 2015b) where THD mutations were exogenously added. In different mice models, it was also possible to observe this TH protein reduction (Tokuoka et al. 2011; Korner et al. 2015).

Second, we assessed TH activity and we detected lower levels of intracellular dopamine and extracellular (supernatant) DOPAC and dopamine in the cell cultures derived from both THD patients when compared to controls.
In the case of mice models; Kobayashi et al. 1995 observed reduced levels of dopamine (DA); adrenaline (A) and noradrenaline (NA). Zhou and Palmiter 1995; Althini et al. 2003 also showed reduced levels of DA and Norepinephrine (NE) or NA, respectively. In the study by Tokuoka et al. 2011 they showed low levels of DA with HPLC analysis. Rose et al. 2015 described reduced levels of L-Dopa, DA, NE and DOPAC but increased levels of 5-Hydroxyindoleacetic Acid (5-HIAA) and 5HIAA/5-HT (5-hydroxytryptamine or serotonin). Finally, Korner et al. 2015 showed low levels of HVA, L-Dopa, DA NE and MHPG but normal levels of neopterin, biopterin and 5-HIAA

Third, we also observed RNA expression of TH gene and we observed a reduction in both THD patients. This was also observed in the mice model from Kobayashi et al. 1995 but Korner et al. 2015 were not able to demonstrate this decreased in relative TH expression.

Considering all, our cell model is a suitable model for Tyrosine Hydroxylase Deficiency modelling, as we observed all the phenotypes that have been described before, together in a one and only model.

Moreover, we were able to find a new neuronal phenotype that was not fully described in the previous models:

First, we detected less TH-immunoreactive cells in both THD derived cell cultures when compared to controls or isogenic ones. This could be due to two main reasons: the first one is that we failed to detect these cells because they express low levels of the protein and therefore, a weaker staining. The second could be a problem either in the proliferation of dopaminergic precursors, or the differentiation into DA neurons. Either way, Rose et al. 2015 addressed this in their mouse model and found no reduction of TH+ cells in DRD mice.

Second, less fiber density was observed in both mutant TH+ neurons. This was somehow also observed in

the models by Rose et al. 2015 and Korner et al. 2015 where they observed les TH intensity by immunohistochemistry in dendrites or projections but at the level of different brain regions; in our case we were able to observe it at neuron level.

Third, we observed a reduction in neurite length in both phenotypes that could be due to a defect in DA neuron development produced by the lack of DA expression and a reduction of the need of synapses.

Fourth, we observed a reduced neuronal arborisation observed in TH+ neurons derived only from B phenotype patient. This could be due to the lower intracellular levels of DA and reduced TH activity which is greater than in A patient. In fact, DA has been already proved as an important neurotrophic factor which could indeed boost a greater and complex morphology (Lieberman et al. 2019).

In line with these last three points of neuronal phenotype; in our THD post-mortem brain (Tristán-Noguero et al. 2016) we described a decreased expression of PSD95 which may suggest a decreased number of synaptic connections and an under expression of NF-H and MAP2 which will imply a reduction in axons and dendritic density.

Last but not least, we observed a reduced axonal TH localization in the soma when compared to the last point in the axon in THD-type A derived DAn but not in the B. This result would suggest a defect in TH transport in our in vitro human model of THD that was already described in the mice model by Korner et al. 2015. They showed more TH immunoreactivity in the substantia nigra than in the striatum and they hypothesized that there could be a transport defect due to a lack in enzyme stabilization or protection by dopamine.

To investigate a little bit how was the gene expression of important genes in Dopaminergic neurotransmitter system and assess if we could observe some differences between both phenotypes we studied the expression of AADC enzyme, dopamine receptors and dopamine transporters:

As mentioned before we observed a reduction in the mRNA TH expression more important in TH B patient derived DAn (p-value <0.01).

We also observed a highly reduced expression in AADC which is the second enzyme in the synthesis of dopamine that converts L-Dopa to DA. We expected to have less AADC expressed as we had lower levels of L-Dopa due to a reduced TH activity, and in the end lower levels of DA.

Regarding dopamine receptors, we observed that D1 was only reduced in B patient derived cultures but

no significance was found. D2DR was reduced in A and B phenotype-derived DAn being more significantly reduced in the B patient (p-value <0.01) when compared to the control. This was already observed in the post-mortem brain study (Tristán-Noguero et al. 2016), even at protein level, where it was described that both receptors were reduced in this B phenotype brain (mainly the D2DR).

On the other hand, Rose et al. 2015 failed to find differences in Dopamine receptors expression in their A phenotype mouse model.

Those differences found between both receptors could imbalance the equilibrium between direct and indirect pathways (Zeiss 2005), therefore producing the parkinsonian signs observed in our patients, which are worse in B phenotype patient (Iravani et al. 2012; Willemsen et al. 2010)

Apart from that, it has been described that D2DR modulates neuronal morphology in the frontal cortex and striatum (Money and Stanwood 2013) and it would make us expect the altered neuronal morphology in means of total neurite length in both patients and neuronal arborisation in B patient TH+ neurons (Li et al. 2015; Lieberman et al. 2019).

Taking into account dopamine transporters we checked DAT and VMAT2 expression:

In the case of DAT, it seems that both patients expressed higher levels (specially A phenotype) but it failed to reach significance. The meaning of this effect could be an adapting process to get the most out of the dopamine that it is possible to produce by this TH defective neurons, and therefore increasing the DA recycle or turnover. This higher expression of DAT has been observed in cocaine exposure during fetal monkey development (Fang and Rønnekleiv 1999) or after the use of methylphenidate in the treatment of ADHD disorder (Wang et al. 2009).

Finally, in the case of VMAT2 it is slightly decreased in both patients, especially in B patient derived Dan. This would be expected as this transporter is in charge of the monoamine loading in vesicles that are then liberated in synapses; and taking into account that we have less dopamine to load, it makes sense to downregulated its expression. It doesn't reach significance maybe because it's a transporter for monoamines and is not only specific to dopamine (Wimalasena 2011).

We then used this model to test different therapeutic approaches in vitro and repeat the same analysis that were used to defined the phenotype to assess the treatment possible effect.

The first treatment assessed was the use of chaperones as it had promising effects in a THD PC12 cell model (Hole et al. 2015).

We tried different doses as we observed a lethal effect in higher concentration doses and we selected finally one that was no lethal (10 um). With this last concentration, we failed to find any major effect in the cell cultures when compared to mock treatment.

What it lacks now is to study in more detail the lethal dosage and try different moments of treatment to

finally adjust the moment and find the best concentration to be able to see an effect.
What we would expect with this treatment would be to observe a better functioning of TH activity and therefore recover or increase DA levels in our cell model in both phenotypes. We could also expect an increased expression in TH protein and RNA levels and maybe in the expression of other DA genes.
Only in the A phenotype we would expect to recover the TH transport defect due to an improvement in the TH stabilization by the chaperone. Finally, we could also see some recovery regarding the neuronal phenotype such as increased fiber density and morphology defects, but taking into account that this chaperone has not been tested before in such a complex model, we should try first.

The second treatment used was a combination of L-Dopa plus carbidopa which is the normal treatment that is given to patients and we wanted to check how our cell model will respond to it.
This treatment consists in giving the L-Dopa which is the product of the reaction performed by TH, which is not working well in THD patients. In addition, carbidopa is given, which is an inhibitor of AADC enzyme (which converts the L-Dopa to dopamine). It is usually given to patients to inhibit the peripheral L-Dopa metabolism and to guarantee that the major part of L-Dopa arrives to the brain.
We decided to test the exact treatment, as it has been described that while A patient responds well to the treatment and has a good outcome, the B patient is not able to and have further problems.
We were afraid that carbidopa could somehow mask the possible effect of the L-Dopa, but it didn't occur.

We test the treatment in DAn derived from one control and both patients. Comparing the cell culture treated vs the mock we observed:

First, we observed and increase in TH+ and Tuj1+ neurons in A phenotype derived cultures that was less observed in B phenotype derived neurons. One one hand, the effect observed in all the neurons (including TH+ neurons) could be due to the known neurotrophic effect of dopamine through regulation of BDNF expression levels (Kueppers and Beyer 2001) . Adachi et al. 2018 and Hasbi et al. 2009 showed that this BDNF regulation could be managed through dopamine receptors. Taking into account that both D1DR and D2DR expression has been found to be reduced in our THD B phenotype DAn will explained the lower effect in B patient cultures On the other hand, the detection of more TH+ neurons exclusively could be due to the TH expression boost itself.

Second, after the treatment with L-dopa + carbidopa we could observed a full recovery of fiber density in the A patient DAn that was not observed in B patient DAn. This could be due to the inability of B patient neurons to respond to this dopaminergic input as it has a reduced expression of D2DR. It has been described that this receptor regulates neuronal morphology in some brain regions (Money and Stanwood 2013) and therefore it could also affect to the area occupied by TH fibers.

Third, an increase in TH expression by WB which was lower in B patient derived DAn. We could hypothesize that the L-dopa or the dopamine obtained by its catabolism binds to TH enzyme stabilizing it and therefore boosting its expression.

Fourth, increased levels of intracellular DA and extracellular DA and DOPAC in all lines (which makes sense as this exogenous L-Dopa that we are giving to cell cultures has to been metabolized by them and therefore led to greater levels of DA and DOPAC).

Fifth, regarding relative mRNA expression analysis we observed an increased in TH expression in both patients after L-Dopa treatment that it was not observed in the control (Dopamine could have an effect also boosting TH expression at the RNA level).
We observed also an increased in AADC enzyme only observed in the control but not the patients (patient basal levels were already lower and with the carbidopa treatment they failed to increase. Although we will expect an upregulation of its expression, as there's more substrate (L-Dopa)).
In the case of Dopamine receptors, both receptors' expression is increased in the control after the treatment and in the D2DR in the A patient, but in the other cases they failed to increase after treatment. This could be due because these pathways are strongly dysregulated, especially in B phenotype cell cultures.
DAT expression was increased after the treatment in the control but not in the patients (maybe they have lost its adaptive effect to get the most out of the available dopamine).
Last but not least, we observed an increase of VMAT2 after the treatment of L-Dopa (we would expect that, as now there's more dopamine to be load in the synaptic vesicles).

In the end we obtained the results we were expecting the A patient derived neuronal cultures fully respond to the L-dopa treatment and the B patient don't respond or respond only partially.
Even though we should repeat these analysis as it had been done only once and check also if those differences are statistically significant (n=3)

To our knowledge this is the first cell model based on iPSCs and therefore we cannot comment about what has been observed in other models. Otherwise in one study (Ishikawa et al. 2016); they describe two neurotransmitter disorders that have been modelled with iPSCs (which are PTPS and DHPR disorders) and they found similar phenotypes as the ones described in our THD cell model: reduced % of TH-positive neurons, less TH stained area (what we have called fiber density), lower TH protein expression and decreased levels of extracellular DA. They also used this model to test different therapeutic approaches and recover most of the phenotypes. All in all, will go in line with what we have observed in our cell model.

As a future prospect, to better understand how the transport defect was occurring in TH A derived DAn we decided to perform an extra experiment with primary neuron culture (rat cortex):
This experiment will consist in transfecting this culture with a construct containing the WT or mutant forms of TH (3 different mutations called A, B1 and B2) fused in N-terminal with dendra2 (linked with a flexible link). Dendra2 is a photoswitchable protein which initially has green fluorescence and with a photoswitcth with UV light turns red.
This way we could trace how is the distribution at the beginning (green) and then how, once is photobleached (turns red), dendra-TH is transported through the neuron; from the soma to the axon and will allow us to study also the defect in B mutations separately.

In summary, this is a new tool that would allow us to better understand the interactions between the presynaptic dopamine depletion, with the mal adaptive receptor responses and the abnormalities in neurons that finally arise to this specific symptomatology of THD that is so different between both phenotypes.
It would be also a valuable platform to test different therapeutic approaches in vitro and luckily find one possible treatment that could addressed both phenotypes.

CONCLUSIONS

THD postmortem brain

- Dopamine is an important neurotransmitter that plays a role not only in the regulation of the synthesis of different dopaminergic enzymes, transporters and receptors but also in other neurotransmitter systems.

- Adds valuable information to our growing comprehension of neurodevelopment and the pathophysiology of movement disorders starting at early fetal stages as it is the case in THD.

Proteomics study

- First study that uses a novel –omic technique in a large cohort of patients with monoamine defects.

- With the descriptive analysis, it was possible to confirm that our population harbours the same biochemical findings that are described for these disorders in the literature.

- The main category of common overrepresented proteins is related to crucial functions in neurodevelopment (plasticity, excitability, synapse maturation and branching).

- We could therefore state that IEM of monoamines are neurodevelopmental disorders and that there should be a change in the paradigm on how these disorders are usually addressed

- We have found several proteins that could be useful biomarkers for treatment and severity prognosis that are specific of disorder such as ApoD, Col6a3, Apo H and OMGP.

- Some of these proteins are expressed peripherally so we should confirm the results already obtain in CSF with plasma to see if we could avoid invasive procedures as the lumbar puncture

IPSCs model

- We have established a new THD model based on iPSCs technology, which recapitulates the disease phenotype. Indeed, upon differentiation, DAn derived from either THD Type A and B-iPSCs showed reduced TH expression and enzyme activity as well as alterations in the expression of key dopaminergic genes such as TH and AADC enzymes, dopamine receptors 1 and 2, DAT and VMAT2, which were not evident in DAn from Ctrl-iPSC.

- Moreover, a new neuronal phenotype that was not described before has been identified. Specifically:
    1. Dopaminergic neuronal culture differentiated from either Type A and Type B THD-iPSCs showed less TH-immunoreactive cells with less fiber density and decrease total neurite lenght compared to DA neuronal culture obtained from Ctrl-iPSC.
    2. A reduced axonal TH localization was observed in THD-Type A derived DAn which was not observed in DAn derived from THD-Type B or Ctrl iPSCs,
    3. A reduced arborisation was found in THD-Type B derived DAn that was not observed in DAn derived from THD-Type A or Ctrl iPSC
- L-Dopa + carbidopa treatment totally reversed the disease and neuronal phenotypes in Type A THD DAn and only partially in Type B THD DAn.

In summary an iPSC model for THD has been developed for the first time that introduces mechanistic insights and open the path to exploring novel therapeutic strategies.

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ANNEX

I. Discovery of Biomarker Panels for Neural Dysfunction in Inborn Errors of Amino Acid Metabolism

This work analyses the mechanism underlaying the neural dysfunction present in MSUD and UCD patients despite the accurate metabolic control. For doing so they studied the expression of different proteins and gene expression identifying a biomarkers panel that could be used for this purpose.

II. Synaptic metabolism: a new approach to inborn errors of neurotransmission

This article introduces the concept of synaptic metabolism (specific chemical composition and metabolic functions of the synapse). It also states the need of an extended definition of inborn errors of metabolism including new mechanisms and categories: disorders of chaperones and autophagy, disorders of the synaptic vesicle, diseases affecting pre-synaptic membranes, disorders of the vesicle pools or receptor trafficking among others.

This specific article and others that were published in the JIMD journal has led to an invitation to participate in the ICIMD (International Classification of Metabolic Diseases) to redefine this metabolic disorders with this new synaptic metabolism approach.

III. Diseases of the Synaptic Vesicle: A Potential New Group of Neurometabolic Disorders Affecting Neurotransmission

This study states the need of evolving from the classical biochemistry point of view of the concept of inborn errors of metabolism towards a more biological understanding.

One example of this is the case of monogenic defects of the synaptic vesicle, especially the ones affecting synaptic vesicles' cycle that could be a new potential group of Neurotransmitter disorders.

IV. Study of a fetal brain affected by a severe form of tyrosine hydroxylase deficiency, a rare cause of early parkinsonism

First study in a unique sample of THD postmortem brain which highlights the importance of dopamine in the regulation of dopaminergic and other neurotransmitter systems as in general brain development. And how this regulation is altered in movement disorders with origin at the embryological state such as THD