FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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Application of Brain Organoids to Study Autism Spectrum Disorder - A Portuguese Cohort

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE PEDIATRIA

Trabalho realizado sob a orientação de:

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05/2020

Application of Brain Organoids to Study Autism Spectrum Disorder - A Portuguese Cohort

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Coimbra, May 2020

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Abbreviations

ABA	Applied Behaviour Analysis Therapy
ADIR	Autism Diagnostic Interview – Revised
ADOS	Autism Diagnostic Observation Schedule
ASD	Autism Spectrum Disorder
CNC	Center for Neuroscience and Cell Biology
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
FMUC	Faculty of Medicine, University of Coimbra, Portugal
GDQ	Global Development Quotient
HP-CHUC	*Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra*
ID	Intellectual Disability
SNIPI	*Sistema Nacional de Intervenção Precoce na Infância*
SCQ	Social Communication Questionnaire
VS	Ventricle structures

Abstract

Introduction: Social impairment, difficulties in communication and interaction, as well as repetitive, restrictive and stereotypical behaviours are some of the core symptoms of Autism Spectrum Disorder (ASD). This lifelong neurodevelopment disorder affects 9.2 in 10000 children in Portugal, there is no cure neither biomarkers to detect it and confirm the diagnosis. Brain organoids, as translatable models, were developed and characterized using staining techniques in order to increase our knowledge on this cerebral disorder.

Objectives: Perform a clinical characterisation of our cohort of ASD patients and explore human brain organoids as a model to assess neurodevelopment.

Materials and Methods: We characterized a cohort of 18 patients from *Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra* by analysing their clinical data on neurodevelopmental, comorbidities, medication, family history, gestation data and Apgar score. ASD diagnosis was based on gold standard instruments (Autism Diagnostic Interview-Revised and Autism Diagnostic Observation Schedule) and an etiologic genetic study in some cases. In the control population we have 19 elements and ASD was excluded based on the Social Communication Questionnaire and another questionnaire was applied to collect data on clinical and neurodevelopment signs. Stem cells from exfoliated deciduous teeth were collected and stored in *Hospital Pediátrico's* biobank. Brain organoids were analysed with three staining protocols and one immunohistochemistry protocol and the resulting images were analysed using *Zen* and *Image J* software, to better understand their conformation.

Results: We were able to fully characterize our ASD population. ASD levels were distributed through the Intellectual Disability levels in an unbalanced way ($p=0.034$; tau kendel b coeficient=0.505) and the weight at birth was related with the number of comorbidities ($p=0.021$). Additionally, it was possible to characterize cryosections from brain organoids, with different kind of staining to identify the structural differences in brain organoids.

Discussion: Our ASD population is greatly varied and this is certainly an advantage when studying a complex disorder that is ASD. A greater number of individuals is required for comparative analysis between different categories of the population. The protocols applied to brain organoids are straightforward and valuable, as they allow for a good characterization of their structures.

Conclusion: We have managed to fully characterize our ASD population and staining protocols have proven to be efficient and practical ways of studying brain organoids' structure during neurodevelopment. This work will lay the groundwork for the development and testing of novel therapeutic strategies.

Keywords: Autism Spectrum Disorder, Brain organoids, Neurodevelopmental disorder

Introduction

Autism Spectrum Disorder (ASD) is a lifelong, neurodevelopmental disorder, which severely affects 9.2 in every 10000 school aged children in Portugal[1]. The first manifestations appear during early childhood, being the most common and specific the social impairment, difficulties in communication and interaction, as well as repetitive, restrictive and stereotypical behaviours[2]. The lack of biomarkers, that would allow for ASD's detection, is an issue as the diagnosis relies solely on clinical data, child development scores and the direct observation of their neurodevelopment and behaviour[3,4]. Currently, there is no precise treatment available for ASD's core features, and interventions are made through symptomatic management[3]. The drugs approved for use in Portugal are risperidone or aripiprazole, when previous psychoeducational intervention fails, to control severe behaviour alterations[3]. And sodium valproate, carbamazepine or topiramate, to diminish emotional lability, disruptive episodes and control impulsiveness[3]. Early intervention programs are an important part of ASD intervention protocol, as they provide useful tools for these children, verbal or non-verbal, to use in their daily life[5]. In Portugal, each child with ASD has the right to receive occupational and speech therapy and psychological intervention, they are also given the opportunity of having teaching plans adapted to their needs and of being integrated in centres specialised in supporting the learning process of ASD students[3]. ASD is also related with a variety of comorbidities, being the most common, intellectual disability (ID), sleep and eating disturbances, language and motor impairment, irritability, disruptive behaviours, epilepsy, attention deficit hyperactivity disorder, anxiety, obsessive-compulsive disorder and depression[2,5]. ASD is not only considered a highly heritable disorder[5,6], related with genetic mutations, for instance, *Copy Number Variations* and monogenic disorders (e.g. fragile X syndrome, Timothy syndrome and Angelman syndrome)[7], but is also influenced by the environment[2]. Environmental factors (e.g. prenatal infections, premature birth and maternal obesity[2,5]) may act as a trigger when genetic vulnerability is present or increase the risk for ASD[2]. Despite the investigation that has been performed thus far, most cases of ASD are considered idiopathic[8]. Each ASD's case is unique and varies significantly between individuals[2]. Besides, the limitation in accessing brain tissue has prevented our ability to determine the mutational effects in neurons and brain development. Animal models, as non-human primates or rodents, arise a great range of ethical issues, as they are not genetically equal to humans and the conclusions reached through them will always have to be tested in humans, in order to validate them[9,10]. Therefore, it is critical to develop realistic models that will advance knowledge on ASD and possible therapeutic strategies.

A breakthrough of 3D human brain organoid development has now allowed to examine high-order brain functions[9]. Patient-derived brain organoids represent a personalized and realistic model to mimic the potential brain alterations underlying ASD[10,11]. Giving the opportunity to test for personalized strategies that could have a therapeutic impact on ASD. Brain organoids are

representative of an early developing brain, approximately in the first trimester of gestation[12]. This model not only allows for the study of the organization of 2 to 3 million neurons[13] in the developing brain structure, but also their function. For example, neurons inside brain organoids are capable of self-assembling Ventricle-like structures (VS), that closely mimic the neural tube formation during embryonic neurodevelopment[14]. Developing brain organoids using dental stem cells as a tissue source is minimally invasive, as these cells are isolated from teeth that have naturally shed or that have been extracted with a previous medical indication. Brain organoids will lay the groundwork to demonstrate whether there are differences in brain development and function between controls and ASD patients, making this a realistic and robust model to study neurodevelopmental diseases.

Objectives

Our work was divided into two parts, that are summarized in the specific goals below:

Part 1) Perform a complete clinical characterisation of a cohort of ASD patients that have donated dental stem cells for this study.

Part 2) Explore human brain organoids as a model to assess neurodevelopment.

Materials and Methods

This work was integrated within the ProTeAN project, funded by the European Commission (Grant #799164) and is being carried out through a collaboration among the Center for Neuroscience and Cell Biology (CNC), the Faculty of Medicine of the University of Coimbra (FMUC) and the *Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra* (HP-CHUC). The collected dental stem cells are stored in the first Portuguese biobank of this type of cells to study neurodevelopmental disorders, at the HP-CHUC. This project has been approved by the Ethics Committee of FMUC (Ref: 121-CE-2017) and CHUC (Ref: CHUC-049-18).

Part 1) Clinical characterization of our cohort of ASD patients

To disseminate our project to society, we developed flyers (Appendix 1) to raise awareness and explaining the objectives of the ProTeAN project. These materials were delivered during medical appointments at HP-CHUC.

The only restriction for the collection of samples is the age of the individuals, as they must have between 5 and 25 years of age. These limits are compatible with the beginning of the shedding of the deciduous teeth, and with the need of extracting "wisdom" teeth, or third molars. The process of teeth collection is summarized in Figure 1.

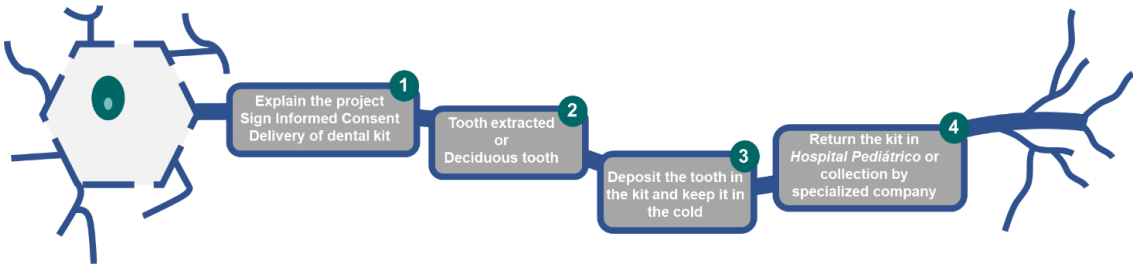

Figure 1 *- Graphic explanation of the process of tooth collection.*

To collect deciduous teeth, a "Tooth Kit" was given to whom showed interest in participating in this study, during the medical appointment with the Neurodevelopmental Pediatrician. The kit consisted in a tube with a specific medium that should be kept by the parents in their domestic fridge (approximately, 4ºC) or in the freezer (approximately, -20ºC), in case of long-term storage. As soon as the deciduous tooth is shed, it should be placed inside the supplied tube and delivered

to the HP-CHUC, or use the contacts in the flyer, in order to schedule a time for the tooth to be collected.

In the case of the wisdom teeth, they were extracted at the Dental Medicine Unity of the Faculty of Medicine, in Coimbra, and were conserved in the same medium and delivered to CNC. As to the ASD population, that needed to have their third molars extracted but could not do it without sedation, an intervention was schedule at the Stomatology Service of HP-CHUC.

An Informed Consent containing the details of the study, developed in accordance with Declaration of Helsinki, Oviedo Convention and Portuguese Legislation, was signed by parents and/or donors, when the dental kit was given or at the time of the dental extraction. Remaining one document with the participant and another stored in HP-CHUC.

To exclude ASD in the control population, with 19 elements, two additional questionnaires were applied, in order to identify those individuals with higher risk of ASD and in need of a specialized assessment. The questionnaires were the standardized Social Communication Questionnaire (SCQ) (Appendix 2) and another that we developed at the HP-CHUC with the goal of collecting data regarding the presence/absence of comorbidities, medication, prenatal and gestation data, Apgar score and neurodevelopment information about acquisition of milestones skills at key ages (age with which they started walking and talking, cephalic perimeter at birth, height at birth and weight at birth) (Appendix 3).

To characterize our ASD population, we analysed their clinical files and information on ASD diagnosis, *Autism Diagnostic Interview – Revised*[15] (ADIR) and *Autism Diagnostic Observation Schedule*[16] (ADOS) scores, as well as functional skills evaluated by Vineland Adaptative Behaviour Scale[17] and Griffiths Scale[18] (Appendix 4), and other clinical data - presence/absence of comorbidities, medication, family history (positive when cases of neurologic or psychiatric disorders were present), gestation data, Apgar score (it was considered as needing life support an Apgar score <7, in the first minute) and neurodevelopment information (age with which they started: walking, talking, controlling sphincters day and night, cephalic perimeter at birth, height at birth and weight at birth).

The diagnosis and ASD evaluation were performed by a very specialized team (neurodevelopmental pediatricians and psychologists) of Autism Unit, that is a reference in Portugal for this medical condition and other neurodevelopmental disorders.

As described in the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*[19] *(*DSM-5), there are three severity levels of ASD, being level 1 the mildest and level 3 the most severe. In order to classify our patients in these 3 categories, we analysed their total score on ADOS[16] (the score is based on cut-offs in the areas of reciprocal social interaction, communication and language deficits). The higher this score is, the greater the clinical severity. The cut-offs used were:

- When used module 1 of ADOS[16] (child with no words or single words, maximum score = 24): score ≤11 = level 1; score ≥12 and ≤18 = level 2; score ≥19 = level 3.
- If module 2 of ADOS[16] (child with phrases, maximum score = 24) was used: score ≤11 = level 1; score ≥12 and ≤18 = level 2; score ≥19 = level 3.
- When module 3 was applied (child with fluent language, maximum score = 22): score ≤9 = level 1; score ≥10 and ≤16 = level 2; score ≥17 = level 3.

When evaluating ID, five severity levels were considered, based on the Global Development Quotient (GDQ) of the Griffiths Scale[18] (the higher the quotient is, the better development; normal range: 100±15[20]), being Borderline level a GDQ between 70 and 79, Mild abnormal level a GDQ between 50 and 69, Moderate abnormal level between 35 and 49, Severe abnormal level between 20 and 34 and Profound level inferior to 20. The GDQ data were crossed with the score on Global Parameter, in Vineland Adaptative Behaviour Scale[17], in order to corroborate the results obtained in Griffiths Scale[18] and confirm ID and Borderline levels. This Global Parameter should be ≤ 70 (approximately, 2 standard deviations below the population mean)[19].

Statistical analysis:

The *Kolmogorov-Smirnov test* was used to test for normality. A *t-test* and a *non-parametrical test* were used to compare between independent samples.

The results were considered statistically significant when $p \leq 0.05$.

This analysis was performed using SPSS™23.0.

Part 2) Explore human brain organoids as a model to assess neurodevelopment

In order to study brain organoids, three staining protocols were tested, to be able to assess its development through their structural organization. The brain organoids used were developed from the control population in order to optimize these protocols.

Brain organoids were embedded in a gelatin solution and then frozen using dry ice. To obtain brain organoids section, we used a cryostat and cut section with a thickness of 10µm for an optimal cell density when imaging. Brain organoids sections were mounted onto superfrost glass slides, dried at room temperature and then stained using the different protocols, having in mind that each of them highlights a specific structure. Nissl Staining allowed us to observe the nucleus, as it stains them in dark blue. Hematoxylin & Eosin Staining is a combination that emphasizes the nucleus through the action of hematoxylin (violet), being the intensity of staining correlated with the quantity of DNA in the nuclei and the time that the sample is in it; and distinguishes the cytoplasm of the nuclei, by conferring it a pink colour. The Luxol Fast Blue Staining is used to stain myelin.

Nissl Staining Protocol is present in Table 1.

Table 1 – Nissl Staining Protocol

xylene	4min
100% ethanol	4 min
95% ethanol	2 min
70% ethanol	2 min
type 1 water	2min
0,5% cresyl violet	6 min
type 1 water	2 min
70% ethanol	1 min
95% ethanol	1 min
100% ethanol	2 min
xylene (xylene until mounting)	5 min

Description of the different steps in Nissl Staining Protocol.

We started with 99,8% ethanol and then diluted it with type 1 water, in order to achieve the different ethanol concentrations. Finally, the sections were mounted using *Permount* mounting medium.

Hematoxylin and Eosin Staining Protocol can be seen in Table 2.

Table 2 - Hematoxylin and Eosin Staining Protocol

NUCLEAR STAINING	**100% ethanol**	1 min
	95% ethanol	1 min
	75% ethanol	1 min
	50% ethanol	1 min
	type 1 water	1 min
	Hematoxylin	2 min
Cytoplasmatic staining	**type 1 water**	1 min
	Scotts top water	1 min
	type 1 water	1min
	Eosin	45 sec
	75% ethanol	1 min
	95% ethanol	1 min
	100% ethanol	1 min
	xylene (xylene until mounting)	3 min

Description of the different steps in Hematoxylin and Eosin Staining Protocol.

The sections were mounted using Richard-Allan Scientific™ medium.

Luxol Fast Blue Staining Protocol is described in Table 3.

Table 3 - Luxol Fast Blue Staining Protocol

Luxol fast blue (1%)	On 60ºC (12-16h)
95% ethanol	
type 1 water	
Lithium	5 sec
70% ethanol	10 sec
70% ethanol	10 sec
type 1 water	
repeat steps	1 time, from step 2
70% ethanol	
eosin	1 min
type 1 water	
cresyl violet	1 min
type 1 water	
95% ethanol	1 min
100% ethanol	1 min
Xylene (xylene until mounting)	5 min

Description of the different steps in Luxol Fast Blue Staining Protocol. When time is not specified, is just needed to dip the slide.

The sections were mounted using Richard-Allan Scientific™ medium.

The resulting images of these staining protocols were collected using Axio Imager Z2 microscope, using a 10x objective. Image analysis was completed using *Zen* and *Image J* software and their respective tools. These brain organoids sections were then analysed in terms of:

- Total area (Figure 2)
- Total perimeter (Figure 2)
- Diameter (Figure 2)
- Number of VS
- VS characterization

All items were measured three times.

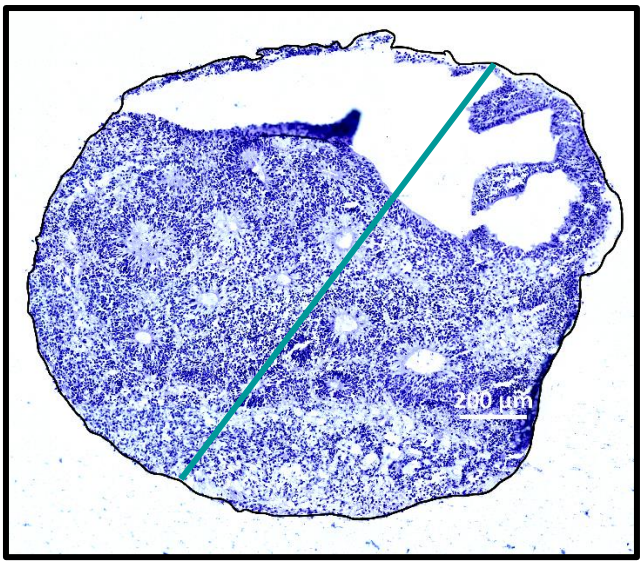

Figure 2 *- Total section perimeter (black line) and area (everything inside black perimeter) and example of total section diameter (green);*

In order to characterize VS, measurements were done with *Image J*. We calculated the area and perimeter of the Loop (Figure 3a) and Ventricle Structure (Figure 3b) and the area of Loop tissue (Figure 3c).

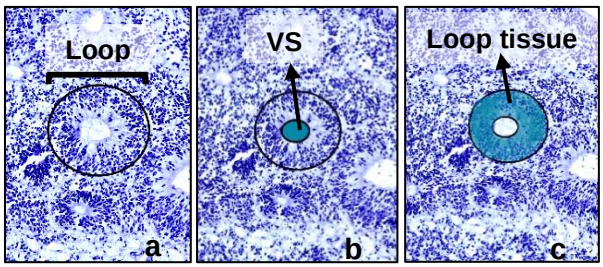

Figure 3 *- a) Loop borders defined corresponding to basal membrane; b) VS area in green, delimitating it is the apical membrane; c) Loop tissue area in green.*

An Immunohistochemistry Protocol (Table 4) was performed, to promote a better understanding on the relation between morphology, showed by the previous Staining Protocols, and neuronal cell function. Using this protocol, it is possible to distinguish neuronal progenitors from more differentiated ones, excitatory neurons from inhibitory ones and early born neurons from late born neurons, depending on the primary antibody used. We used sections from brain organoids of different maturation states, 35 days, 60 days and 258 days, for this protocol.

Table 4 - Immunohistochemistry Protocol

Wash the brain organoids with PBS once and fix using PFA 4%	1h30min
Put the brain organoids in 30% sucrose solution when section it on cryostat	Overnight
Section it directly to the slide in the cryostat (10µm)	
Wash with PBS	Two times
Permeabilize the cells with 0,5% Triton in PBS	15min
Wash with PBS	Two times
Incubate with 3% BSA in PBS	1h
Incubate with 1^{ry} antibody in 3% BSA	1h at room temperature or overnight at 4ºC (in a humidified box)
Wash with PBS	Two times
Incubate with 2^{ry} antibody	At room temperature, for 2h, in the dark
Wash with PBS	Two times
Stain with Hoechst/DAPI (1µm/ml)	5min, in the dark
Wash with PBS	Two times
Let it dry	
Mount with Dako Mounting Medium	
Seal the coverslip	With nail polish
Go to microscope	

The antibodies used are listed in Table 5.

Table 5 - Antibodies used for Immunohistochemistry.

Primary Antibody	Structure highlighted	Secondary Antibody
Anti - Nestin (rabbit) 1:500	neuronal progenitors	**Alexa Fluor 488 goat anti-rabbit** 1:200
Anti - NeuN (mouse) 1:100	mature neurons	**Alexa Fluor 568 goat anti-mouse** 1:200
Anti - MAP2 (guinea pig) 1:500	mature neurons	**Alexa Fluor 647 goat anti-guinea pig** 1:200
Anti - TBR1 (rabbit) 1:300	deep layer neurons	**Alexa Fluor 488 goat anti-rabbit** 1:200
Anti - SATB2 (mouse) 1:200	upper layer neurons	**Alexa Fluor 568 goat anti-mouse** 1:200
Anti - GAD67 (mouse) 1:100	inhibitory neurons	**Alexa Fluor 568 goat anti-mouse** 1:200
Anti - VGLUT (guinea pig) 1:5000	excitatory neurons	**Alexa Fluor 647 goat anti-guinea pig** 1:200
Anti - GFAP (mouse) 1:400	astrocytes	**Alexa Fluor 568 goat anti-mouse** 1:200
Anti - Sox10 (rabbit) 1:250	oligodendrocytes	**Alexa Fluor 488 goat anti-rabbit** 1:200

Antibodies used for Immunohistochemistry, with the respective concentration used, and structures that each highlight.

Results

Part 1

ASD population characterization:

Our ASD population was composed of 18 individuals, being the average current age of 9.3 years old, of which 66.7% were males. The average age of ASD diagnosis was 3.4 years old. Two of them are twins, two are cousins in first degree and the other are not related. We were able to divide them into ASD levels: we had 5 individuals in level 1, 10 individuals in level 2 and 2 individuals in level 3 (Table 6). Only in one individual was not possible to analyse his ASD level.

Table 6 - Number of individuals in each ASD level.

ASD level		1	2	3
ADOS	Module 1 (no words or single words)	n=5	n=6	n=2
	Module 2 (phrases)	n=0	n=2	n=0
	Module 3 (fluent)	n=0	n=2	n=0

Only one individual did not have a previous genetic test and in another two it was not possible to gather this information. One individual presented the *FMR1* DNA test. The other 14 presented *FMR1* DNA test along with an Array CGH test. Eight out of the 15, with genetic tests, had a normal result, with no mutations found. Seven individuals had genetic variants identified, that are specified in Table 7.

Table 7 - Genetic variants and respective relative syndromes.

Genetic Mutations	Related syndrome
***CACNA1C* (CNV gain - dup 12p13.33)**	Timothy Syndrome
SHANK3	Phelan – Mcdermid Syndrome
CNV loss - 3q26.31q26.32x1	-
CNV gain – dup 14q24.2	-
***SLC6A8* mutation c.1519_1543del**	X-Linked Creatine Deficiency
***FMR1* expansion (>200 CGG)**	Fragile X Syndrome
MECP2 mutation	Rett Syndrome

We compiled all the comorbidities present in our population (Table 8). Briefly, in four individuals it was not possible to collect information on possible comorbidities (except for the presence/absence of ID, that was evaluated in all the 18 individuals), seven individuals had no comorbidities present, and as to the remaining ones, three presented one comorbidity, two of them presented two comorbidities (ID + Eating problems; ID + Psychomotor agitation), one had three comorbidities (ID + Eating disorder + Cardiopathy) and one had four comorbidities (Eating disorder + Motor abnormality + Sleep disturbances + ID). Psychopathological Disorders, Epilepsy and Gastrointestinal Problems were not reported in our population.

Table 8 - Types of comorbidities.

		Number of individuals (respective percentage, %)
Comorbidities	Intellectual Disability	7 (38.9%)
	Sleep Disturbances	1 (7.1%)
	Eating Disorder	3 (21.4%)
	Motor Abnormalities	1 (7.1%)
	Others: - Idiopathic Juvenile Arthritis - Cardiopathy - Psychomotor Agitation	1 (7.1%) 1 (7.1%) 2 (14.3%)

Regarding ID specifically (Table 9), we considered different severity degrees. In our population, only Mild abnormal and Borderline perturbation were present. What concerns Borderline degree, there was one individual and in Mild abnormal ID there were seven.

Table 9 - Different levels of ID.

Intellectual Disability	Number of individuals
Normal	10
Borderline	1
Mild	7

Only one individual is currently doing pharmacological treatment with Risperidone.

In terms of therapies (Figure 4), it was only possible to collect information on 15 individuals. 93.3% of them were on Speech Therapy; 73.3% were on Occupational Therapy; 46.7% were on Educational Support; 46.7% were on *Sistema Nacional de Intervenção Precoce na Infância* (SNIPI); 13.3% Sensory Integration Therapy; 13.3% in Psychomotricity Therapy; 6.7% had Psychological Support; 6.7% had Physiotherapy; and 6.7% on Applied Behaviour Analysis Therapy (ABA).

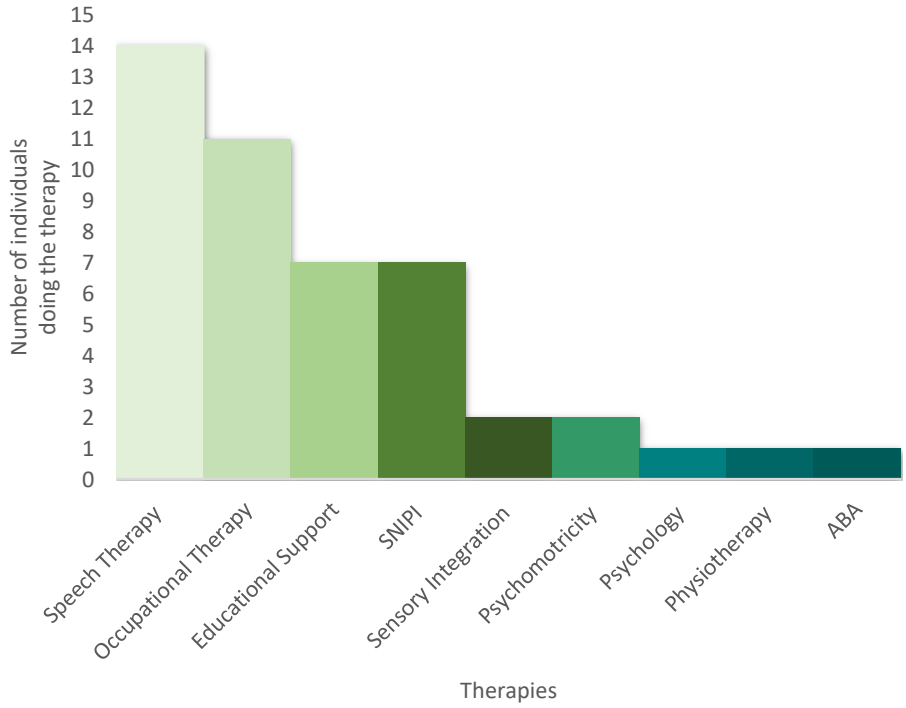

Figure 4 *– Graphic explanation on the number of individuals taking a specific therapy.*

Family history (Figure 5) was another topic of evaluation in our population, and only in two cases (11%) was not possible to have information on this. In eight cases it was negative, no history of other cases of neurologic or psychiatric disorders. In the other eight, the history was positive for ASD or Humour Perturbation or Dementia or Epilepsy or Intellectual Disability or Learning Difficulties.

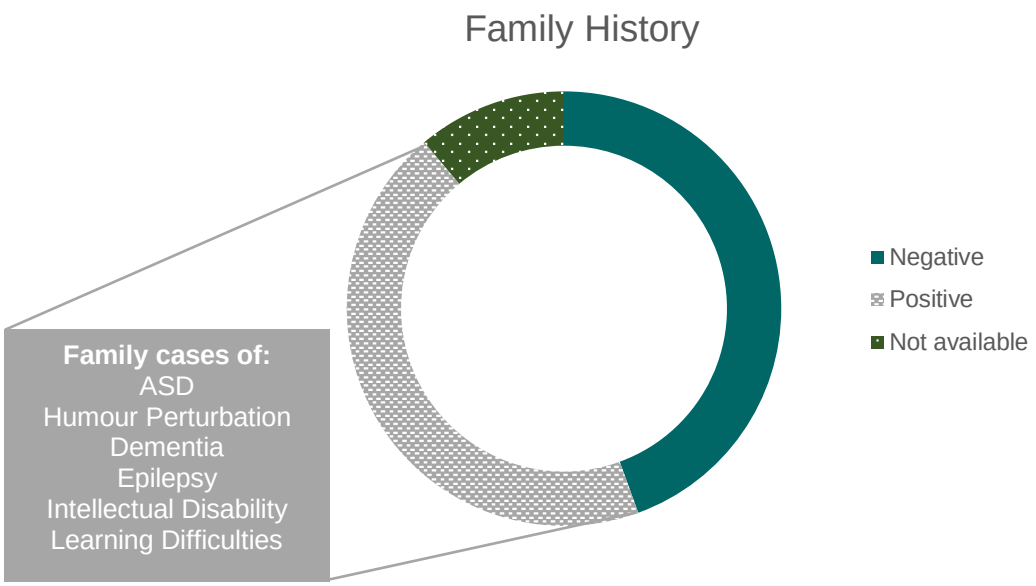

Figure 5 *– Graphic showing the percentage of individuals in each category of the Family History, and the different kinds of Psychiatric and Neurologic diseases present when positive.*

The pregnancy data, collected on all the individuals except for one, allowed us to see that two individuals had history of gestational diabetes and two of preterm labour threatening and the other 13 had no complications. There were three premature births (two at 35 weeks of pregnancy and one at 30 weeks of pregnancy).

In terms of extrauterine life adaptation, two of them had Apgar score <7, at the first minute, which means they needed life support care. But at the fifth minute all of them had this score superior to 7.

In Table 10 are summarized the median age, in months, with which our population started walking, said their first words, had their sphincters controlled by daytime and night, and the average results on cephalic perimeter, stature and weight.

Table 10 - Neurodevelopment data collected.

Data	Median Age (months)	Number of individuals with information
Start Walking	14 [IQR=4]	17
First Words	24 [IQR=8]	15
Sphincter controlling by day	42 [IQR=12]	15
Sphincter controlling by night	48 [IQR=18]	12
Data collected at birth	**Average Results ± SD**	**Number of individuals with information**
Cephalic Perimeter	34.1 ± 1.1 cm	16
Stature	46.5 ± 1.2 cm	16
Weight	2939.4 ± 243.2 g	16

IRQ = Interquartile Range; SD = Standard Deviation.

Based on the statistical analysis done, it seems that the different ASD levels distribute themselves in a different way through the various levels of ID (p=0.034; tau kendel b coeficient=0.505). And that the weight at birth was related with the number of comorbidities of these children, as individuals with zero or one comorbidities had inferior average weight, at birth, than the ones with two or more comorbidities (p=0.021).

Part 2

After collecting the resulting images of each staining protocol application, except for Luxol Fast Blue Staining Protocol, it was possible to analyse them.

In Figure 6 is possible to see the measurements that have been done in the brain organoids.

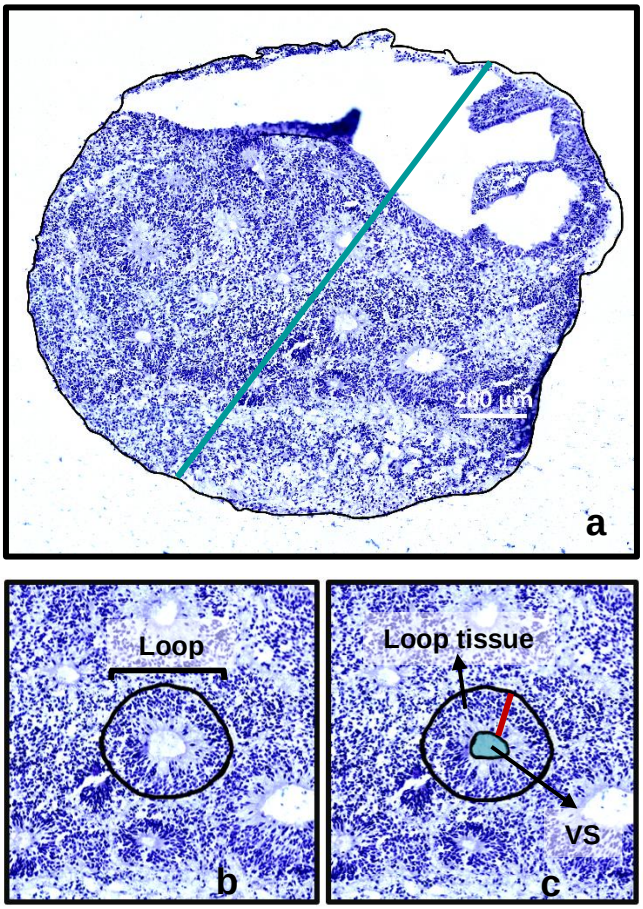

Figure 6 *– Example of measures taken on Image J. a) Total section perimeter and area (everything inside black perimeter) and example of total section diameter; b) Loop perimeter and area (everything inside black perimeter); c) VS perimeter and area (in green) and Loop tissue diameter (red line). The same process was used for Nissl and Hematoxylin and Eosin images.*

- **Hematoxylin and Eosin Staining**

In this brain organoid section stained with Hematoxylin and Eosin (Figure 7) we can see the VS areas.

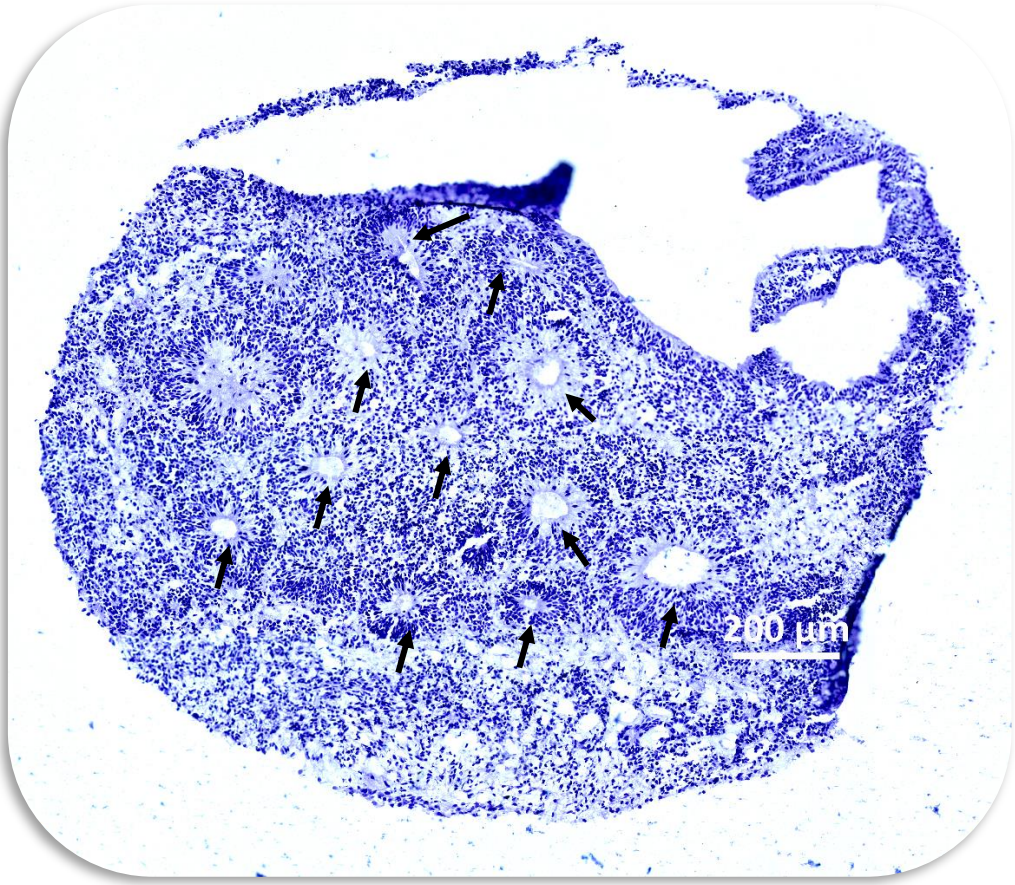

Figure 7 *– Example of brain organoid section, of 35 days of maturation, with Hematoxylin and Eosin Staining, here we can observe 11 VS areas (arrows).*

In this section (Figure 7), the total area of the section was 2000162.8 µm^2 and the perimeter was 5504.3 µm. The results of all the VS measured were gathered and the average loop area was 30811.3 µm^2, the average loop perimeter was 659.8 µm, the average VS area was 1513.8 µm^2, the average VS perimeter was 148.5 µm and as to the loop tissue are, the average was 27461.8 µm^2, the average loop diameter was 72.2 µm. These results are collected in Table 11 and in Figures 8 and 9. Details of the measures taken to each VS individually can be seen in Figure 10.

Table 11 - Results of figure 7 analysis.

Structures	Average results (µm and µm² when area)
Total section area	2000162.8
Total section perimeter	5504.3
Total Loop area	30811.3
Loop perimeter	659.8
Total VS area	1513.8
VS perimeter	148.5
Loop tissue area	27461.8
Loop diameter	72.2

Results: 11 VS were analysed. Here is possible to see the area and perimeter of the section and the average area and perimeter of all the parts that constitute a VS.

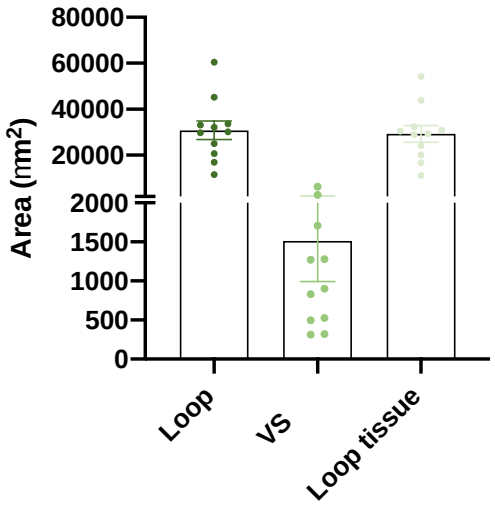

Figure 8 *– Graphic illustration of mean total Loop area, mean total VS area, and mean total Loop tissue area.*

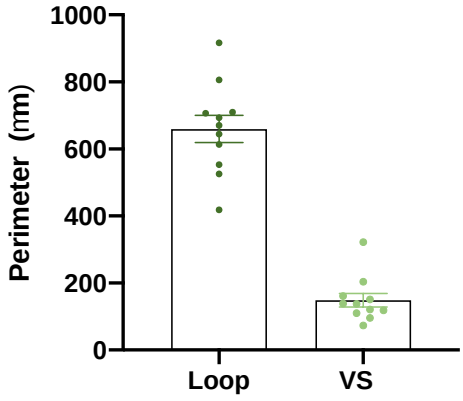

Figure 9 *- Graphic illustration of mean total Loop perimeter, mean total VS perimeter.*

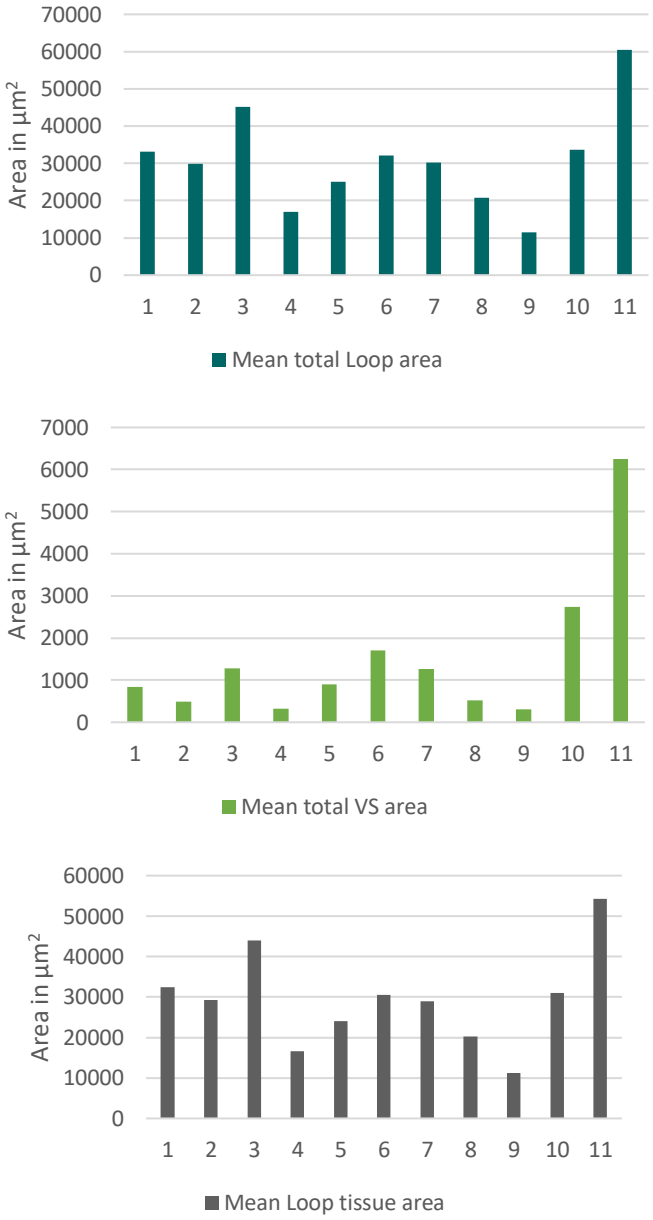

Figure 10 *– Graphic representation of each VS in figure 6, through different parameters. These mean results reflect the mean of the 3 measurements done for each VS in terms of Total Loop area, Total VS area and Loop tissue area.*

- **Nissl Staining**

In this brain organoid section stained with Nissl (Figure 11) we can see the VS areas.

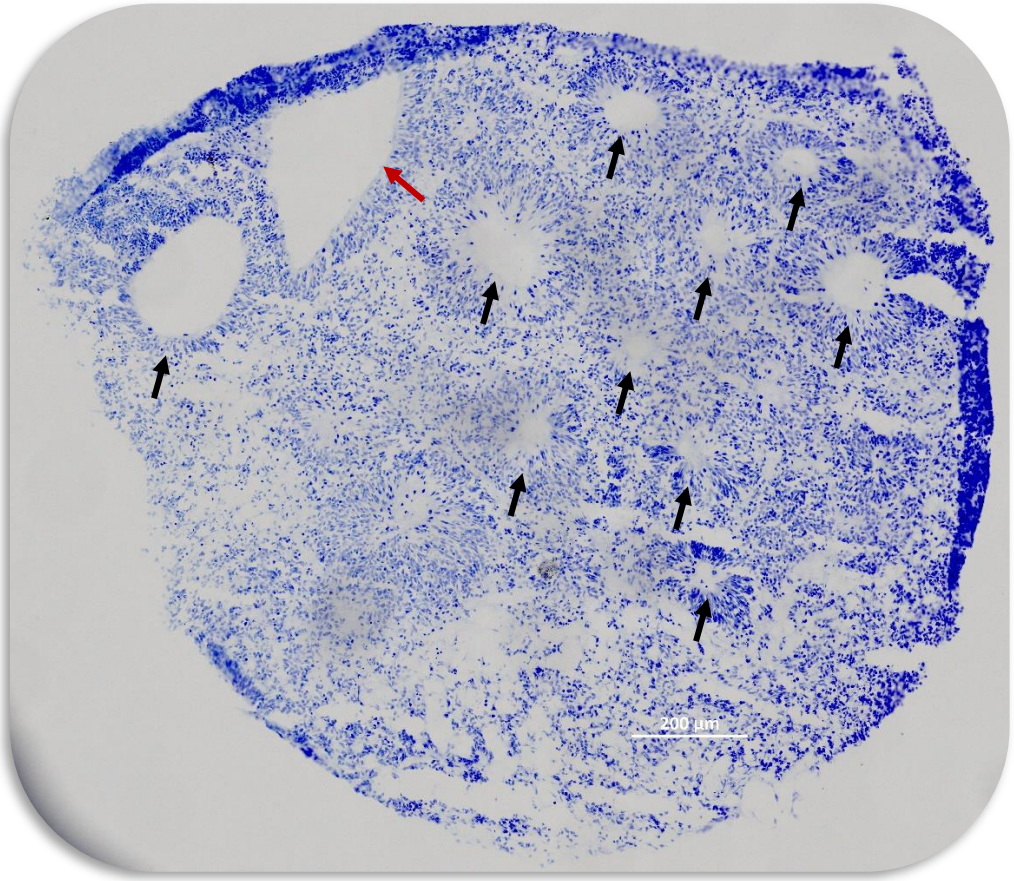

Figure 11 *- Example of brain organoid section, of 35 days old, with Nissl Staining, here we can count 11 VS areas (arrows); VS with red arrow was not possible to analyse.*

In this section (Figure 11), the total area of the section was 1856449.3 µm^2 and the perimeter was 5366.2 µm. The results of all the VS measured were gathered and the average loop area was 37523.7 µm^2, the average loop perimeter was 728.7 µm, the average VS area was 5408.6 µm^2, the average VS perimeter was 235.3 µm and as to the loop tissue are, the average was 32115.1 µm^2, the average loop diameter was 66.8 µm. These results are collected on Table 12 and in Figures 12 and 13. Details of the measures taken to each VS individually can be seen in Figure 14.

Table 12 - Results of figure 11 analysis.

Structures	**Average Results** (µm and µm^2 when area)
Total section area	1856449.3
Total section perimeter	5366.2
Total Loop area	37523.7
Loop perimeter	728.7
Total VS area	5408.6
VS perimeter	235.3
Loop tissue area	32115.1
Loop diameter	66.8

Results: 10 VS were analysed. Here is possible to see the area and perimeter of the section and the average area and perimeter of all the parts that constitute a VS.

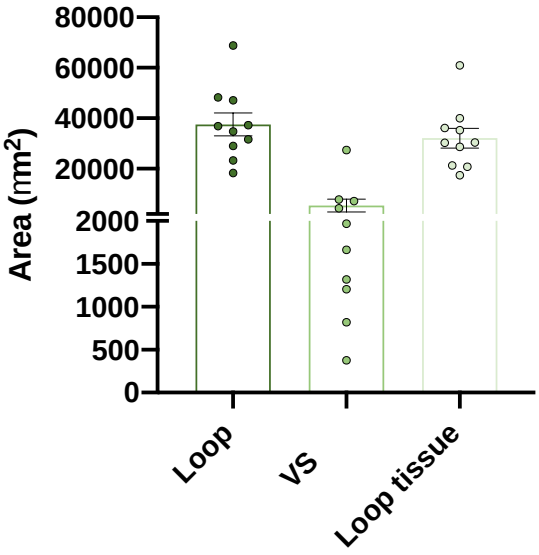

Figure 12 *- Graphic illustration of mean total Loop area, mean total VS area, and mean total Loop tissue area.*

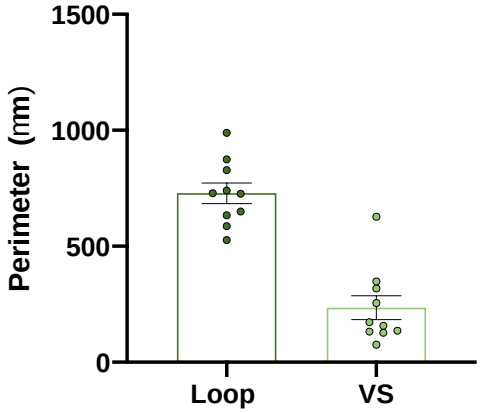

Figure 13 *- Graphic illustration of mean total Loop perimeter, mean total VS perimeter.*

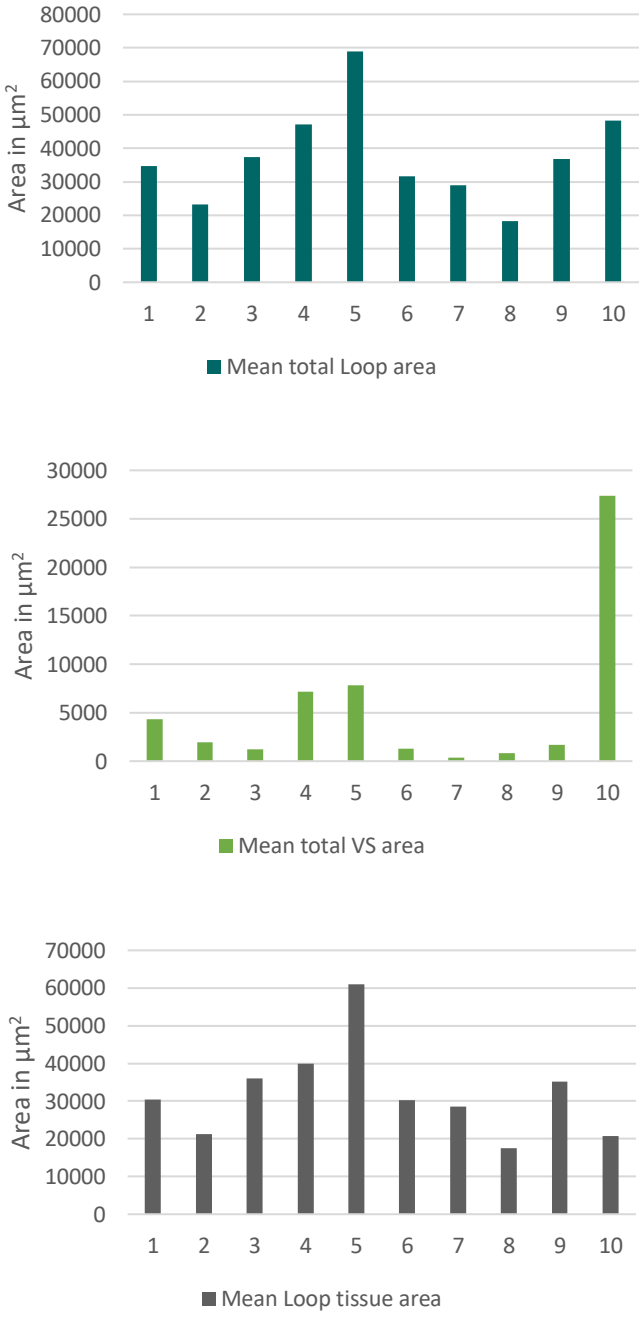

Figure 14 *- Graphic representation of each VS in figure 10, through different parameters. These mean results reflect the mean of the 3 measurements done for each VS in terms of Total Loop area, Total VS area and Loop tissue area.*

Discussion

We have a great variability in our population of ASD patients, with representation of several syndromic forms of ASD[21]. We can state that all our patients had a diagnosis of ASD, that followed the most updated guidelines, in Portugal.

The number of individuals of our patient population is modest, that limited the analyses in terms of statistical power and difficulted the interpretation of the results achieved. From our cohort, ASD´s levels appear to correlate positively with ID´s levels (higher levels of ASD correlate with higher levels of ID) and that individuals with zero or one comorbidities seemed to have inferior average weight, at birth, than the ones with two or more comorbidities. As to the relation between ASD and ID levels, it is said that lower IQ levels are related with higher severity of ASD[22], which is concordant with our result. Specifically regarding the possible relation between birth weight and comorbidities, our results seem to diverge from what is described in literature (although not in ASD populations), as for example low birth weight (between normal range) has been associated with epilepsy[23] and also seems to relate with inferior cognitive performance[24], as well as with a poor metabolic profile (lower insulin sensitivity)[25]. Furthermore, as our population is small it may not accurately represent the general ASD population. Therefore, no conclusions are possible to withdraw from these results. This could be surpassed by increasing the number of individuals in our ASD population.

ID is described in literature as one of the most common comorbidities in ASD patients, appearing in approximately 45% of the cases[2], and this is observed in our population as seven individuals are affected with this perturbation (38.9%).

In our point of view, it is important to notice that all ASD individuals were engaged in multiple therapies and having educational support, as advised in Portuguese guidelines[3], which shows the importance of offering these opportunities, as they bring benefits to the individual growth and facilitate the individual integration in society[2].

Our future work will include completing the collection of data from the control population, in order to fully characterize this cohort and to rule out the possibility of them having ASD. This will be essential in this innovative study as it will permit the comparison between brain organoids from typical neurodevelopment control participants and brain organoids from ASD participants, as well as the populations between themselves. Our final purpose would be to find a connection between this spectrum of neurodevelopmental disorders' phenotype and the specific morphological and functional features of the brain, that can diverge from a normal pattern to a pathological one.

Regarding brain organoids' characterization, we validated three protocols for staining that were straightforward and will be easy to use them in future experiments. They can bring us important information in terms of brain development and the neuronal organization. Optimization is required

in terms of the sectioning process to overcome difficulties in obtaining intact sections, especially when cutting with a thickness of 10 µm. The acquisition of the section onto the slide was the most challenging as it tended to roll up. Another difficulty faced was the fact that the gelatin previously used to embed the brain organoids was also amenable to staining, making it difficult to distinguish it from the brain organoids. So, in the future, it is recommended to embed the brain organoids in Optimal Cutting Temperature compound – OCT compound, which is not permeable to the staining. We were able to optimize the staining process by assuring that all gelatin was removed from the slides.

The Luxol Fast Blue Staining Protocol stains myelin structure and this was not visible in our brain organoids' sections, which is expected as we used a brain organoids of 35 days of maturation and myelin is not present in such early stage of development[26]. Regarding the other two staining protocols, we concluded that the Hematoxylin and Eosin Staining protocol worked best as the images obtained were clearer and allowed a better contrast between structures. With these protocols, it was very interesting to observe that each VS is different, in terms of shape, area and dimensions, showing the different stages of development and conformation.

Due to technical issues, it was not possible to acquire the images from the Immunohistochemistry protocol that was performed. A more conclusive and complete characterization of brain organoids will be possible when combining those results with the ones presented in this study, as we will be able to combine the structural results from the visible staining protocols, with cell-specific information, from the immunostaining results.

Our results in terms of brain organoids' characterization indicate that they will certainly allow for interesting comparisons between brain organoids from our control and ASD populations and even between different stages of maturation.

As future direction, with this complete characterization of our ASD population, that has great variability in terms of etiology and environment factors, we will corelate their phenotypic profiles with the structure and function of brain organoids. This will be a key point in our work as it will allow a personalized approach, particularly in terms of possible therapies.

Conclusion

Currently, there are no therapeutic strategies for the core features of ASD. Understanding more about brain development and ASD will pave the way for novel targets for ASD therapeutics. This study using brain organoids will certainly contribute to improve the knowledge on ASD, as they represent the most advanced human-based cellular model to study neurodevelopmental diseases and allow for mature brain functions at levels previously unattained.

Future work includes the characterization of the control cohort and the application of the validated protocols to establish comparisons between brain organoids derived from patients and controls and to correlate the histological issues in brain organoids and neurodevelopmental main diagnosis and associated comorbidities. We will as well increase our cohort to enhance the statistical power of this work.

This work constitutes a great example of translational research in Coimbra, establishing a bridge between the clinical knowledge of a health unity of excellence in this area, *Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra* (HP-CHUC), and the most advanced research in laboratory, led in this work by Center for Neuroscience and Cell Biology (CNC).

Acknowledgments

I am grateful to Professora Doutora Guiomar Oliveira, without her guidance and expertise this work would not be possible.

To Doutora Catarina Seabra, who has inspired me to challenge myself and to always do better, I express my gratitude.

I thank Dr. Frederico Duque, Dra. Cátia Café and Dra. Alexandra Oliveira for the support they gave me and for the availability with which they welcomed me.

To Doutor João Peça, I must thank for the opportunity of being part of this interesting project.

I express my gratitude to Ana Rafaela Oliveira and Mariana Laranjo, for all the help, all the advices and kind words.

To my friend Martina, who has always been there for me with a word of encouragement, thank you for believing in me and for your faithful friendship. To Maria João, I thank her dedication, help and kindness that were fundamental to conclude this work. Thank you, Ana Sofia and Joana, for your companionship and friendship.

To my family, especially my parents and my brother, and to Guilherme, I thank all the support, their unconditional belief in me and their love.

I thank my cousin Sofia for being my inspiration and the reason I embraced this project.

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Appendix

Appendix I – Flyer for Project Dissemination

ProTeAN

As perturbações do neurodesenvolvimento afetam cerca de 3% da população mundial. O estudo ProTeAN pretende aumentar o conhecimento acerca destas perturbações para uma medicina mais precisa e personalizada.

Recolha de amostras
É essencial o acesso a amostras biológicas para estudar as perturbações do neurodesenvolvimento, por isso, estamos a recolher dentes de leite e do siso que contêm células estaminais.

Quem pode participar
Crianças e jovens entre os 5 e os 25 anos de idade.

O que vamos estudar
Com as células dentárias estamos a desenvolver organóides cerebrais, conhecidos como "mini-cérebros", onde podemos estudar as alterações no desenvolvimento de neurónios.

ProTeAN **Produção e Teste de neurónios e organoides cerebrais humanos: modelos avançados para o estudo de doenças do neurodesenvolvimento**

Referência das Comissões de Ética:
121-CE-2017
CHUC-049-18

Como participar?

O recrutamento será feito através de consulta médica na Unidade de Neurodesenvolvimento, do Hospital Pediátrico de Coimbra.

Deve ler e assinar um Consentimento Informado e ser-lhe-á entregar um *Kit Dentário* (para recolha de dentes de leite) ou será encaminhado para uma consulta de Medicina Dentária (para recolha de dentes do siso).

Caso não seja portador de perturbações do neurodesenvolvimento, poderá participar como controlo, contactando-nos através do email **info.protean@gmail.com**.

Colaboradores:

CHUC HOSPITAL PEDIÁTRICO
BioBanco CIFC Hospital Pediátrico
CENTRO DE INVESTIGAÇÃO E FORMAÇÃO CLÍNICA
FMUC FACULDADE DE MEDICINA UNIVERSIDADE DE COIMBRA
MARIE CURIE ACTIONS
CNC CENTER FOR NEUROSCIENCE AND CELL BIOLOGY UNIVERSITY OF COIMBRA PORTUGAL

Para mais informações acerca deste estudo, contactar: ***info.protean@gmail.com***

Appendix II – SCQ

Social Communication Questionnaire

AutoScore™ Form

Michael Rutter, M.D., F.R.S., Anthony Bailey, M.D.,
Sibel Kazak Berument, Ph.D., Catherine Lord, Ph.D. and Andrew Pickles, Ph.D.

Publicado por WESTERN PSYCHOLOGICAL SERVICES

Data: _____ / _____ / _____

Nome: _____

Data de Nascimento: _____ / _____ / _____

Idade Cronológica: _____ Y _____ M Género ☐ F ☐ M

Preenchido por: _____

Grau de Parentesco: _____

Local da Avaliação: _____

Examinador: _____

Instruções

Obrigada por ter disponibilizado o seu tempo para responder a este questionário. Por favor responda a cada uma das questões fazendo um círculo à volta do **SIM** ou do **NÃO**.

Algumas das questões colocadas descrevem vários padrões de comportamento que se relacionam entre si. Faça um círculo à volta do **SIM** nestas questões sempre que **QUALQUER UM** dos comportamentos referidos se encontrou presente NÃO seu/vosso filho/a nos **ÚLTIMOS 3 MESES**.

Mesmo que não tenha a certeza se determinado comportamento se encontrou presente ou não no período de tempo referido, não deixe de responder **SIM** ou **NÃO** a todas as questões, de acordo com o que pensa.

1. Tem frases? SE **NÃO** avance para a questão 8	SIM	NÃO
2. É capaz de manter um diálogo adequado (i.é, uma conversa que envolva uma discussão e uma troca de ideias)?	SIM	NÃO
3 Alguma vez utilizou ou repetiu palavras/frases estranhas de uma forma exaustiva e repetitiva (sempre da mesma maneira), fossem palavras/frases que tivesse ouvido dizer ou que tivesse inventado?	SIM	NÃO
4 Alguma vez fez perguntas ou afirmações socialmente inapropriadas (ex. fazer perguntas de foro pessoal/íntimo ou comentários constrangedores a/sobre alguém)?	SIM	NÃO
5 Alguma vez inverteu a utilização dos pronomes numa frase (i.é, dizer *tu* ou *ele/ela* em vez de *eu*)?	SIM	NÃO
6 Alguma vez utilizou palavras aparentemente inventadas por si ou utilizou uma forma metafórica de dizer as coisas (ex. referir-se a *vapor* como *chuva quente*)?	SIM	NÃO
7 Alguma vez repetiu a mesma palavra/frase de modo exaustivo e repetitivo (sempre da mesma maneira) ou lhe pediu para o fazer?	SIM	NÃO

8 Alguma vez realizou uma actividade ou tarefa de um modo particular ou numa determinada ordem ou insistiu que outras pessoas realizassem determinados rituais (ex. insistir que alguém abrisse e fechasse a porta repetidamente)?	SIM	NÃO
9 Considera que ele/ela revela uma expressão facial adequada a determinada situação?	SIM	NÃO
10 Alguma vez utilizou a mão de outras pessoas para segurar ou alcançar objectos ou como se fosse um prolongamento do seu próprio corpo (ex. utilizar o dedo de outra pessoa para apontar ou colocar a mão de outra pessoa NÃO puxador da porta para a abrir)?	SIM	NÃO
11 Alguma vez manifestou interesse por algo ou por alguma actividade que considerasse estranha ou que parecesse estranha aos olhos de outras pessoas (ex. marcas de automóveis, canos de esgoto ou horários)?	SIM	NÃO
12 Alguma vez lhe pareceu estar mais interessado/a em partes de um brinquedo ou objecto do que propriamente em utilizá-lo ou em brincar com ele do modo que seria esperado (ex. girar as rodas de um carro em vez de brincar com ele)?	SIM	NÃO
13 Alguma vez manifestou um interesse que tivesse considerado ser de uma intensidade invulgar, embora fosse algo apropriado à sua idade e aos gostos do seu grupo de pares (ex. dinossauros, comboios)?	SIM	NÃO
14 Alguma vez revelou um interesse invulgar em fixar o olhar, tocar, ouvir, lamber ou cheirar objectos ou pessoas?	SIM	NÃO
15 Alguma vez manifestou maneirismos motores ou fez movimentos estranhos com as mãos ou dedos (ex. fazer *flapping* ("abanar as mãos") ou abanar os dedos ou as mãos à frente dos olhos)?	SIM	NÃO
16 Alguma vez manifestou movimentos estranhos como, por exemplo, andar às voltas ou pular repetidamente?	SIM	NÃO
17 Alguma vez se auto-agrediu de forma deliberada (ex. morder-se, bater com a cabeça na parede)?	SIM	NÃO
18 Tem um objecto que insiste em levar sempre consigo, que considere invulgar?	SIM	NÃO
19 Tem algum amigo/a preferido/a ou um/a melhor amigo/a?	SIM	NÃO

Nos comportamentos seguintes, tenha em conta o período de tempo entre os 4 e os 5 anos de idade. Pode facilitar recordar-se de eventos chave, como o início de frequência no jardim de infância, mudanças de casa, época natalícia ou outros eventos que são particularmente marcantes para a sua/vossa família.
Se o seu/vosso filho/a ainda não tiver 4 anos de idade cronológica, refira-se ao seu comportamento nos últimos 12 meses.

20. Quando tinha 4-5 anos, alguma vez falou consigo só para ser amável (em vez de ser para pedir algo)?	SIM	NÃO
21. Quando tinha 4-5 anos, alguma vez o/a imitou espontaneamente ou a outras pessoas ou as vossas acções (ex. aspirar, jardinagem, consertar coisas)?	SIM	NÃO
22. Quando tinha 4-5 anos, alguma vez apontou espontaneamente para as coisas à sua volta para as mostrar (ou apontava apenas como forma de pedir algo)?	SIM	NÃO
23. Quando tinha 4-5 anos, alguma vez usou gestos, para além do apontar ou puxar pela sua mão, de forma a demonstrar o que queria?	SIM	NÃO
24. Quando tinha 4-5 anos, acenava com a cabeça para dizer que "sim"?	SIM	NÃO
25. Quando tinha 4-5 anos, acenava com a cabeça para dizer que "não"?	SIM	NÃO
26. Quando tinha 4-5 anos, costumava olhar directamente para sua cara enquanto fazia coisas ou falava para si?	SIM	NÃO
27. Quando tinha 4-5 anos, sorria em resposta ao sorriso de outra pessoa?	SIM	NÃO
28. Quando tinha 4-5 anos, alguma vez ele/ela lhe mostrava coisas de forma a obter a sua atenção?	SIM	NÃO
29. Quando tinha 4-5 anos, costumava partilhar coisas consigo, sem ser comida?	SIM	NÃO
30. Quando tinha 4-5 anos, alguma vez procurava partilhar consigo a alegria de estar a fazer algo que lhe provocava prazer?	SIM	NÃO
31. Quando tinha 4-5 anos, alguma vez procurou confortá-lo/a quando estava triste, magoado/a ou doente?	SIM	NÃO
32. Quando ele/ela tinha 4-5 anos, quando queria alguma coisa ou precisava de algo, olhava para si e usava sons ou palavras acompanhadas por gestos para obter a sua atenção?	SIM	NÃO
33. Quando tinha 4-5 anos, apresentava uma variedade normal de expressões faciais?	SIM	NÃO
34. Quando tinha 4-5 anos, participava espontaneamente e tentava imitar acções em jogos sociais (ex. brincar à roda ou brincar à apanhada)?	SIM	NÃO
35. Quando tinha 4-5 anos, tinha jogo simbólico, ou seja, brincava ao *faz-de-conta*?	SIM	NÃO
36. Quando tinha 4-5 anos, interessava-se por outras crianças aproximadamente da mesma idade, que não conhecia?	SIM	NÃO

37. Quando tinha 4-5 anos, reagia positivamente quando outras crianças se aproximavam dele/dela?	SIM	NÃO
38. Quando tinha 4-5 anos, se chegasse a uma divisão da casa e começasse a falar para ele/ela, sem o/a chamar pelo nome, costumava olhar para si ou prestar atenção ao que lhe estava a dizer?	SIM	NÃO
39. Quando tinha 4-5 anos, alguma vez brincou de forma imaginativa com outra criança de uma maneira que o/a pai/mãe conseguia perceber que as crianças compreendiam o que cada uma estava a imaginar?	SIM	NÃO
40. Quando tinha 4-5 anos, participava cooperativamente em jogos de grupo (ex. escondidas, mata)?	SIM	NÃO

Appendix III – Questionnaire for Controls

Código

Questionário para colheita de amostras controlo - ProTeAN

Nome: _____

Sexo: F ☐ M ☐

Data de nascimento: ____ /_____ / _____Data de preenchimento: ____ / _____ / _____

1. Qual foi a duração da gravidez (semanas)? (Se possível consultar o boletim de grávida e boletim de saúde infantil e juvenil)

_____

2. Teve algum problema/doença na gravidez? (Se possível consultar o boletim de grávida)

Não ☐ Sim ☐

Se sim,
qual/ais?_____

_____

3. Fez vigilância durante a gravidez, cumprindo todas as consultas estipuladas? (Se possível consultar o boletim de grávida e boletim de saúde infantil e juvenil)

Não ☐ Sim ☐

Se não,
porquê?_____

_____

4. Durante a gravidez, o bebé teve algum problema no seu desenvolvimento? (Se possível consultar o boletim de grávida e boletim de saúde infantil e juvenil)

Não ☐ Sim ☐

Se sim,
qual/quais?_____

_____

5. Teve alguma complicação no parto? (Se possível consultar o boletim de grávida e boletim de saúde infantil e juvenil)

Não ☐ Sim ☐

Se sim,
qual?_____

_____

6. Índice Apgar: (Se possível consultar o boletim de saúde infantil e juvenil)

1º minuto:_____ 5º minuto:_____ 10º minuto:_____

7. Peso ao nascer? (Se possível consultar o boletim de saúde infantil e juvenil)

_____

8. Comprimento ao nascer? (Se possível consultar o boletim de saúde infantil e juvenil)

_____

9. Perímetro cefálico ao nascer? (Se possível consultar o boletim de saúde infantil e juvenil)

_____

10. Com quantos dias teve alta da maternidade, o/a seu/sua filho/a? (Se possível consultar o boletim de saúde infantil e juvenil)

_____

11. O/A seu/sua filho/a teve algum problema nos primeiros dias de vida? (Se possível consultar o boletim de saúde infantil e juvenil)

Não ☐ Sim ☐

Se sim,

qual/quais?_____

_____

12. Em criança/Atualmente, o seu/sua filho/a apresentou/apresenta alguma doença/problema? (Se possível consultar o boletim de saúde infantil e juvenil)

Não ☐ Sim ☐

Se sim,qual/quais? _____

13. Com quantos meses é que o seu filho(a) começou a andar sozinho(a)? _____meses

14. Quantos meses tinha o seu filho(a) quando disse as primeiras palavras? _____meses

15. Quantos meses tinha o seu filho(a) quando disse a primeira frase? _____meses

16. Tem alguma preocupação com a linguagem do seu filho?

Não ☐ Sim ☐

Se sim, qual?_____

_____

17. Tem alguma preocupação com o comportamento do seu filho(a)?

Não ☐ Sim ☐

Se sim, qual?_____

_____

18. Tem alguma preocupação sobre como o seu filho(a) se relaciona com as restantes crianças?

Não ☐ Sim ☐

Se sim, qual?_____

_____

19. Tem alguma preocupação em relação à aprendizagem do seu filho(a) na pré-escola/escola?

Não ☐ Sim ☐

Se sim, qual?_____

_____

20. Frequenta alguma consulta hospitalar?

Não ☐ Sim ☐

Se sim, porquê?_____

_____

21. O seu/sua filho/a tem andado doente?

Não ☐ Sim ☐

Se sim, que doença?_____

_____

22. O seu/sua filho/a tem tomado algum medicamento/na última semana/dias?

Não ☐ Sim ☐

Se sim,

qual/ais?_____

_____

Appendix IV – ADOS, ADIR, Vineland Adaptative Behaviour Scale, Griffiths Scale

Brief description of these scales and tools to assess ASD.

Autism Diagnostic Observation Schedule (ADOS)[16]**:** Catherine Lord and her colleagues have published it in 1989[16] and later it has been revised. It is a standardized and structured tool, guided by the investigator, with the objective of in 30 to 45 minutes evaluate the individual capacity of social interaction, communication, play and use of materials in an imaginative way. It has four modules, each adapted to the level of expressive language of the individual (module 1 – child nonverbal; module 2 – child with phrases; module 3 – child with fluent phrases; module 4 – used for teenagers/adults with fluent phrases). Its accurate application is investigator dependent.

Autism Diagnostic Interview-Revised (ADIR)[15]**:** Consists of a structured interview directed to the parents or caretakers of children and adults with mental age ≥ 18 months that are suspected of having ASD. It was elaborated by C. Lord, M. Rutter e A. Le Couter in 1994[15]. It is divided in five parts that include questions about communication, social development, play, behaviour, and its problems, as well as open questions. In order to evaluate social interaction, communication, language and behaviour. It requires trained professionals to apply it.

Vineland Adaptative Behaviour Scale[17]**:** Published in 1984 by Sparrow S.S., Balla D.A. e Cicchetti D.V.[17]. Through the responses given by the parents or caretakers it is possible to assess adaptative behaviour in children ≤ 18 years. It consists of three parameters: Communication, where three domains are covered - written, expressive and receptive -, Socialization, here is included interpersonal relationships, leisure and play and coping skills, and Daily Living Activities in three domains, personal, community and domestic. Through an indirect approach it is also possible to assess fine and gross motor skills and maladaptive behaviour.

Griffiths Scale[18]**:** It was published in 1954, revised in 1984 and its author is Ruth Griffiths and it was adapted and translated to Portuguese[18]. The objective of this scale is to measure the global development of the individual. Evaluates gross motor skills through, for example, postural control, balance and coordination. As well as the level of independence for daily living activities and the child's capacity of interaction with others. Assesses the receptive and expressive language, the fine motor skills and the visual-spatial capacities. Moreover, it evaluates the child's practical reasoning. It is used in children who are or have metal age ≤ 8 years old.