Sidonie Irene Lourenço Monteiro

**Identification of Copy Number Variation by array-CGH in
Portuguese Children and Adolescents Diagnosed with
Autism Spectrum Disorders**

**Identificação de Variações no Número de Cópias por array-
CGH em Crianças e Adolescentes Portugueses com
Diagnóstico de Perturbações do Espectro Autista**

março, 2019

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Mestrado Integrado em Medicina

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Doutora Sofia Dória Príncipe dos Santos Cerveira

E sob a Coorientação de:

Dr. Miguel Jorge Santos O. Ferreira Leão

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Ciências médicas e da saúde

TÍTULO DISSERTAÇÃO/MONOGRAFIA (riscar o que não interessa)

Identification of Copy Number Variation by array-CGH in Portuguese Children and Adolescents
Diagnosed with Autism Spectrum Disorders

ORIENTADOR

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COORIENTADOR (se aplicável)

Miguel Jorge Santos O. Ferreira Leao

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Faculdade de Medicina da Universidade do Porto, 09/03/2019

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*Ao meu irmão Cristiano,
Engrenagem de toda esta aventura pela Medicina.*

Identificação de Variações no Número de Cópias por Array-CGH em Crianças e Adolescentes Portugueses com Diagnóstico de Perturbações do Espectro Autista

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Introdução: As perturbações do espectro autista (PEA) afetam inúmeras crianças tendo uma prevalência estimada de 1%. A Hibridação Genómica Comparativa em arrays (array-CGH) oferece uma sensibilidade acrescida na identificação de anomalias cromossómicas submicroscópicas e é considerada o teste de primeira linha no diagnóstico de PEA. O principal objetivo deste estudo foi a identificação de Variações no Número de Cópias (CNVs) em pacientes com PEA e avaliação da relevância da técnica de array-CGH no diagnóstico etiológico da PEA.

Métodos: Foram selecionados 253 doentes com diagnóstico de PEA pela consulta de neurogenética para realização de array-CGH (Agilent 4x180K microarrays). Foram usadas bases de dados públicas para a classificação dos CNVs e de acordo com “American College of Medical Genetics Standards and Guidelines”.

Resultados: 3,56% (9/253) dos CNVs foram classificados como patogénicos. Quando foram incluídos CNVs provavelmente patogénicos, a taxa aumentou para 11,46% (29/253). Foram identificados alguns CNVs aparentemente não correlacionados com a PEA presente no doente. Atendendo a uma correlação fenótipo-genótipo, estabeleceram-se dois grupos: um grupo incluía os CNVs, de acordo com dados da literatura, com associação conhecida a PEA (23 CNVs presentes em 22 crianças) e outro, com CNVs aparentemente sem relação conhecida a PEA (10 CNVs presentes em 7 crianças). Em 18 casos, realizou-se ainda Sequenciação de Nova Geração (NGS) com painéis para PEA, sendo detetado em 10 pelo menos uma variante em heterozigotia, patogénica ou de significado incerto.

Conclusão: Os resultados do nosso estudo estão de acordo com a literatura, destacando-se a relevância do array-CGH como teste genético de primeira linha no diagnóstico de PEA. O nosso estudo reforça ainda a necessidade de uma complementaridade entre array-CGH e painéis de NGS ou sequenciação de todo o exoma no diagnóstico etiológico de PEA.

Palavras-chave: Hibridação Genómica Comparativa em arrays; Copy Number variation (variação no número de cópias), Sequenciação de Nova Geração; perturbações do espectro autista.

Identification of Copy Number Variation by array-CGH in Portuguese Children and Adolescents Diagnosed with Autism Spectrum Disorders

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Background: Autism spectrum disorders (ASD) affects many children with an estimated prevalence of 1%. Array-CGH (Comparative Genomic hybridization) offers significant sensitivity for the identification of submicroscopic chromosomal abnormalities and it is considered the first-tier genetic testing in ASD. The main objective of this study was to describe the usefulness of array-CGH in the etiologic diagnosis of ASD.

Methods: 253 patients admitted to a neurogenetic outpatient clinic and diagnosed with ASD were selected for array-CGH (4x180K microarrays). Public databases were used for classification and in accordance to the American College of Medical Genetics Standards and Guidelines.

Results: 3,56% (9/253) of CNVs were classified as pathogenic. When likely pathogenic CNVs were considered the rate increased to 11,46% (29/253). Some CNVs apparently not correlated to the ASD were also found. Taking into account a phenotype-genotype correlation the patients were divided in two groups. One group according to previous literature includes all the CNVs related with ASDs (23 CNVs present in 22 children) and another with those apparently not related with ASD (10 CNVs present in 7 children). In 18 patients, a Next Generation

Sequencing (NGS) panel were performed. From these in 10 were identify at least one heterozygous pathogenic or variant of uncertain significance.

Conclusion: The results of our study are in accordance with the literature, highlighting the relevance of array-CGH as the first-tier genetic test in the ASD population. Our study reinforces the need for complementarity between array-CGH and NGS panels or whole exome sequencing in the etiological diagnosis of ASD.

Keywords: Array Comparative Genomic Hybridization; Copy number variation; Next sequencing generation; Autistic Spectrum Disorders;

Introduction

Autism spectrum disorders (ASD) are clinically heterogeneous as a part of a neuropsychiatric syndrome. ASD are estimated to be prevalent among 1% of the general population ¹.

Individuals with ASD may present anomalies that range from cognitive disorders to physical malformations or dysmorphia. Lack of communication, impairment in social interactions and stereotyped behaviours are some of the manifestations of autism. The need for special care can be restrictive to the lives, with DSM-5 being able to differentiate the necessary support levels, from 1, where some support is required, to level 3, with important impairment and support necessities ². Therefore, an early diagnosis helps to provide the affected individuals with valuable coping strategies, such as enhancing their social engagement and reciprocity, to assure a timely appropriate genetic counselling and to enable families with informed reproductive options ³.

Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) provides the basis for the diagnosis of ASD. DSM-5 includes Asperger syndrome, a childhood disintegrative disorder and pervasive developmental disorder within autism spectrum disorders. The criteria

are broader in the interest of diagnosing at an earlier stage. Disorders are now seen as a continuous spectrum, which varies between mild, moderate and severe ².

Scientific evidence suggests that is one of the neurodevelopmental disorders that is most influenced by genetic background, resulting from monogenic or polygenic factors ⁴. However, until recently, a definite etiology could be identified for only approximately 10% of individuals with ASD and in spite of the disease's many possible causes, 90% of patients have “idiopathic autism”⁵.

In the past, cytogenetic aberrations were identified using karyotyping analysis. However, enlarged genome screening methodologies, such as array-CGH, are now considered the gold standard technology to be used as the first-tier diagnostic test in the investigation of ASD. *De novo* pathogenic variants and copy number variations (CNVs) associated with ASD risk genes are also most effectively identified using next generation sequencing (NGS) in multigene panels or exome sequencing and microarray technologies, respectively ⁶.

Once CNVs are detected by array-CGH, it is important to determine their clinical significance by a systematic evaluation. Deletion of a single gene can cause the disease by haploinsufficiency, the remaining copy of the gene being unable to produce a normal phenotype ⁷. The majority of the CNVs gains or losses presents more than one gene affected. The content should undergo an accurate investigation for documented and relevant clinical information, like dosage sensitivity in the interval ⁸.

As for other CNVs with unclear clinical significance, low penetrance and variable expressivity of the phenotype may complicate the analysis and genetic counselling ⁹. Publicly available CNV databases, like the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER), assist in making decisions about the clinical significance of imbalances detected by microarrays.

Research and reporting of data from array-CGH is important to define the etiology of autism and to identify new syndromes, although today there is still a relevant pathophysiological gap between the findings of array-CGH and autism.

In the present work, array-CGH was used to identify CNVs in a cohort of 253 ASD patients. The main objective of this study was to identify pathogenic or likely pathogenic CNVs that could be associated/explain with the phenotype.

Materials and Methods

A retrospective review of data was performed using our array-CGH database performed in the Department of Genetics of the Faculty of Medicine, University of Porto, Portugal, over a 5-year period, between 2012 and 2017. We selected all the patients with an array-CGH test performed and a diagnosis of ASD established by a clinical geneticist, a developmental pediatrician or a child psychiatrist. The database review allowed us to select a total of 253 cases.

Array-CGH was performed using Agilent SurePrint G3 Human Genome 4x180K according to the manufacturer's recommendation, Version 7.5, 2016 (Agilent Technologies, Santa Clara, CA). These microarrays contain approximately 180 000 probes with a 13kb average probe spacing. Results were analysed using Cytogenomics software (Agilent Technologies, Santa Clara, CA) with versions from 2.0 to 4.0.3.12. The arrays nomenclature was described according to ISCN (2016) and using Human Genome build 19.

The following databases were used for the analysis: DGV (<http://dgv.tcag.ca>), DECIPHER (<https://decipher.sanger.ac.uk>), OMIM (<https://www.omim.org>), ClinGen (<https://www.clinicalgenome.org/>), Orphanet (<https://www.orpha.net/consor/cgi-bin/index.php>), related literature and Pubmed (<https://www.ncbi.nlm.nih.gov>).

CNVs were classified as pathogenic, benign, VOUS (Variant of Unknown Significance), and VOUS likely pathogenic or likely benign, in accordance with the American College of Medical Genetics Standards (ACMGS) and Guidelines ⁸. Genetic study of the progenitors was carried out when the clinical geneticist found it relevant for posterior prenatal counselling. Associations between CNVs and clinical data attended the ACMGS and guidelines for interpretation ^{8,10}. We interpreted the findings in the light of clinical information provided by the referring doctor. As such, the clinical data on the request form was used for clinical data ⁷. The statistical analysis was performed using IBM SPSS Statistics 24.0. The study was conducted with the formal approval of by the CHSJ (Centro Hospitalar de São João, Porto) Ethical Committee.

Results

From the general database we selected 253 cases referred with the diagnosis of ASD. Pathogenic or likely pathogenic CNVs were detected in a total of 29 (11,46%) cases. From these, 9/33 (27,2%) were classified as pathogenic and 24/33 (72,7%) as likely pathogenic. However, it was not possible to establish an association between all of these pathogenic CNVs and autism. According to the literature, some CNVs are related with other diseases and so, at least apparently, they could not justify the presence of an ASD. Therefore, we analysed two groups of patients: One group that according previews literature includes all the CNVs related with ASDs (23 CNVs present in 22 children) and another with those apparently not related with ASD (10 CNVs present in 7 children). Some cases have more than one CNVs detected (Figure 1). In the group with CNVs related with ASD, 14 were classified as likely pathogenic and 9 as pathogenic (Table 1). Within the group with CNVs apparently not related with ASD only likely pathogenic or pathogenic for recessive diseases were found (Table 2).

In 18 patients, an NGS panel (for Autism, X-linked mental retardation or Epileptic Encephalopathies) were performed. From these in 10 were identify at least one heterozygous pathogenic variant (Table 1 and Table 3).

According to Table 1, cases 12, 19 and 21 (with pathogenic or likely pathogenic CNVs), turned out normal results in the NGS.

Discussion

According to our results, the array-CGH can be a useful test in neurodevelopmental disorders, namely in ASD. We achieved a total detection rate of 11,46% pathogenic and likely pathogenic variants. Our rate is comparable with previous reports, by Kharbanda *et al* (2014), that usually show a diagnostic yield round 10% ⁷.

Not all these CNVs, were unequivocally considered the cause of the ASD phenotype, some were considered probably pathogenic (10 CNVs found in 7 children). More studies will be necessary in the future in order to determine the significance of these variants and confirming or not the pathogenicity of the CNVs.

Moreover, it is difficult to be sure that some of these variants apparently unrelated to the patient's reason for referral were not correlated with ASD. Besides we did not always have access to the parents' data. Studying parents is important for determining the heritability of CNVs, but both parents need to be available for the analysis and this can be sometimes difficult.

Neurodevelopment disorders are usually accompanied by dysmorphia and other pathologies like epilepsy that also explain the results found in this group (table 2) ^{11,12} . Some studies suggest that the array-CGH is relevant for the identification of autism when children have associated dysmorphic features ¹³. One of these children, case 8 (table 2), has two likely

pathogenic CNVs, and the additive effect of these alterations might also contribute for the etiology.

In the group that includes all the causative CNVs related with ASD, we classified 9 CNVs (3,56%) as pathogenic in 9 children, and 14 CNVs (5,14%) as likely pathogenic, in 13 children. The frequency of pathogenic CNVs found in our study (3,56%) is lower than those found in other reports ⁷ but only when the CNV found was coincident with a well-known syndrome or involves a gene or genes clearly related with ASD the classification given was pathogenic, the remaining were classified as likely pathogenic. Our databases contained also cases that were sent before the DSM-5 had been released (2013), so the criteria for the ASD classification might not be always the same. This could explain, at least in part, this lower frequency.

From the 22 cases included in the table 1 (one patient presents two different CNVs) we selected a few to be discussed in more detail:

CASE 1

The array-CGH technique was used to assess a 6-year-old male child with ASD, showing a 1p32.2 deletion (151KB) that contained the Disabled 1 (*DAB1*) gene. The *DAB1* gene plays an important role in the *reelin* pathway (OMIM 600514). Reelin serves as a ligand for the very-low-density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor-2 (ApoER2) ¹⁴. The gene facilitates the correct migration of neurons within the developing brain ¹⁴ and regulates the synaptic function. The function of Reelin may depend on the phosphorylation of *DAB1* expressed in selectively neurons like Purkinje neurons. Additionally, the variations in *DAB1* function might contribute to make one genetically susceptible to autism, supporting the view that a defect in the Reelin signalling pathway might be a predisposing factor for ASD ¹⁵.

More recently, Sánchez-Sánchez *et al.*, using iPSC-derived neural progenitor cells (NPCs) from one patient with nonsyndromic ASD, suggested an abnormal interplay between Reelin–DAB1 and mTORC1 signalling pathways and further support a multihit model of nonsyndromic ASD risk, in which a coincidental occurrence of disrupting variants in convergent molecular pathways may contribute to ASD ¹⁶.

To evaluate the inheritance of this CNV, progenitors were studied by quantitative polymerase chain reaction (qPCR) and the technique showed that the male progenitor is a carrier of the same microdeletion. However, it is not clear if haploinsufficiency of *DAB1* gene may have an isolated role in the pathophysiology of autism, making it difficult to advise parents on this matter.

Although the deletion was also present in the healthy progenitor considering the possibility of variable penetrance and the information available in the literature this CNV was classified as likely pathogenic.

CASE 3

The array performed in a 5-year-old child with moderate ASD showed a 1q21.1q21.2 duplication (2738KB). This region involves 24 genes (*LOC728989*, *PRKAB2*, *PDIA3P*, *FMO5*, *CHD1L*, *BCL9*, *ACP6*, *GJA5*, *GJA8*, *GPR89B*, *GPR89C*, *PDZK1P1*, *LOC200030*, *NBPF11*, *FLJ39739*, *PPIAL4B*, *PPIAL4A*, *NBPF14*, *PPIAL4D*, *PPIAL4F*, *NBPF15*, *NBPF16*, *PPIAL4E*, *LOC64516*) and was classified as a pathogenic CNV. The region is coincident with the 1q21.1 duplication syndrome (OMIM#612475) with a minimal size of approximately 1.35Mb and according to the literature is associated with autism ¹⁷. The syndrome is also associated with other common phenotypic features such as mild facial dysmorphism, congenital heart defects (tetralogy of Fallot), macrocephaly, intellectual disability or other neurodevelopmental disorders ¹⁷ (ORPHA:250994).

According to the clinical information this child has moderate ASD. One of the most common features is the presence of macrocephaly and it has been suggested that the *HYDIN2* gene is dosage-sensitive and could be responsible for this phenotypic anomaly¹⁸. Our patient does not have macrocephaly reinforcing previous data about the relevance of the duplication of *HYDIN2* gene in the usual phenotype of 1q21.1 duplication syndrome. Previous data also report an increased risk of schizophrenia, so the child should be maintained on surveillance¹⁹. Additionally, this duplication has also been associated with epilepsy. Recently, Gourari *et al*, published a case report of a 10-year-old girl with a low-functioning autism spectrum disorder and focal motor epilepsy²⁰.

The qPCR analysis performed on the parents revealed that the apparently healthy male progenitor was a carrier of the same microduplication. Incomplete penetrance of microduplication 1q21.1 eventually explained by genetic modifiers was suggested by Mefford *et al.* (2008)¹⁷. Consequently, genetic counselling for this duplication, namely in the prenatal context is not straight forward.

CASE 6

The array-CGH performed on a 10-year-old female with ASD showed a deletion with 247KB in 3p26.3. This CNV is included in the 3p deletion syndrome region (OMIM#613792) but only with the *CHLI* gene present (OMIM#607416). The *CHLI* gene has high expression levels in the central and peripheral nervous systems²¹ and have a role in uncoating clathrin-coated synaptic vesicles²². Haploinsufficiency of this gene might result in cognitive deficits²¹. The distal deletion of chromosome 3p25 is associated with low birth weight, congenital heart disease and intellectual disability.

The 3p deletions associated with intellectual deficits are invariably a deletion with approximately 1Mb. This interval contains a few neurodevelopmental genes including *CHLI*,

but the *SRGAP3* gene is considered the major determinant of mental retardation in distal 3p deletions²³. Considering the role of *SRGAP3* gene is not surprising the normal intellectual development in our patient. Since this deletion only involves *CHLI*, a less severe phenotype is expected in our patient. The analysis of both parents by qPCR showed that the deletion was inherited from his healthy father who does not manifest ASD. So, this CNV was classified as likely pathogenic.

Additionally, NGS panel of 116 genes associated with ASD were performed. The result showed a variant of uncertain pathogenicity: c.2981G>A (pArg994Gln) in the exon 3 of *RAI1* gene. This variant was inherited from his healthy mother. Neither the patient or his mother showed any Smith-Magenis syndrome (SMS) phenotype, which is associated with pathogenic variants or microdeletions involving the *RAI1* gene located at 17p11.2.

Further studies will be needed for clear perception of the effects of the mutation present in the patient. However, it is known that *RAI1* gene mutations and autism are positively correlated and could constitute the main clinical presentation²⁴. In fact, about 10% of all the SMS patients, carry an *RAI1* mutation responsible for the phenotype²⁵. So, these studies suggest *RAI1* as a candidate gene in patients with autistic manifestations or social behavioural abnormalities. Our conclusion was that the *RAI1* mutation is the most likely cause of the phenotype.

CASE 7

The array-CGH was performed on a 2-year-old male with ASD, coloboma of the optic nerve and myopia. The result showed a deletion in the chromosome 5, involving the p15.2 region (178KB). This deletion includes the *CTNND2* gene (OMIM604275) which is expressed early in the development and works as a neuronal specific protein. The deletion found is within the 5p deletion critical region²⁶. However, the size is much smaller than the deletions usually found in individuals with the classical phenotype of Cri-du-Chat Syndrome. This means that

may not be expected the most severe phenotype associated with 5p deletions, namely, the presence of intellectual disability.

According to the literature, the *CTNND2* deletion is associated with different phenotypes, such as schizophrenia, severe autism and myopia ²⁷. Our patient presents the last two.

The deletion was identified by qPCR in his healthy father, so this CNV was classify as likely pathogenic.

CASE 12

The array was performed on a 17-year-old male with ASD, polyphagia and macrosomia.

The array-CGH showed a 217Kb deletion in 11q23.3 including the *ARHGEF12* and *GRIK4* genes. The *GRIK4* gene belongs to the GRIK family, with a role in the glutamate receptor pathway and being widely expressed in the brain. Schizophrenia and ASD were reported in cases associated with *GRIK4* overexpression and namely in duplications ^{28,29}. It is suggested an increasing of *GRIK4* expression causes a persistent circuit disequilibrium that alters the main amygdala outputs ³⁰, which might explain the associated disorders, like schizophrenia. Despite that, it is more difficult to understand the pathogenic significance of *GRIK4* loss of function. Indeed, most of the studies are related with *GRIK4* duplication. In a study performed in patients with chromosomal rearrangements, the analysis of the genes disrupted (located in or proximal to the breakpoints) suggested candidate genes associated with psychiatric illness, namely schizophrenia and bipolar disorder. One of the most promising candidates was in fact the *GRIK4* gene ³¹. No recent studies were found in literature focusing the haploinsufficiency of this gene. In ClinVar database only two cases involving isolated *GRIK4* gene were described but with no relevant clinical records.

The study of the progenitors showed a *de novo* deletion. The CNV was classified as likely pathogenic.

CASES 13 AND 14

An array was performed on two 2-year-old male boys (not family related) both with ASD (cases 13 and 14) and mental retardation (case 13).

The array-CGH of both patients shows a 443KB deletion affecting 15q11.2 region. The genes included were *TUBGCP5* (OMIM 608147), *CYFIP1* (OMIM 606322), *NIPA2* (OMIM 608146), *NIPA1* (OMIM 608145) e *WHAMML* and overlaps the *BP1-BP2* microdeletion region. Several patients with mental retardation, behavioural changes (including ASD) and dysmorphism were described with CNVs in this region³². When the deletion is present, affecting the four genes previously mentioned, compulsive behaviour and lower intellectual ability are common features³³. In a recent review of 200 patients with 15q11.2 BP1-BP2 microdeletion, ASD was found in 27% of the cases³⁴.

The 15q11.2 microdeletion is in fact one of the most common cytogenetic abnormalities observed in patients with ASD.

In these cases, a family study using qPCR is recommended in order to understand if the deletion is *de novo* or inherited. The mother of patient 14 was proved to be a carrier of the microdeletion and had a previous child with ASD from a previous relationship. The woman apparently does not have ASD. The parents of patient 13 were not available.

Both CNVs were classified as pathogenic.

CASE 15

The patient was an 18-year-old male with ASD, short stature and microcephaly.

The result of the array-CGH showed a 16p11.2 duplication with approximately 217Kb. The region involves the *ATXN2L*, *TUFM*, *ATP2A1*, *CD19*, *SPNS1*, *LAT*, *SH2B1*, *RABEP2*, *NFATC2IP* genes. This CNV overlaps the 16p11.2 microduplication syndrome (OMIM614671), although with a smaller size. The habitual size of 16p11.2 duplication

encompass an 593Kb interval approximately. Microdeletions and microduplications at 16p11.2 have been associated in 1% of cases of ASD ³⁵. Patients with this duplication syndrome are typically underweight, with a body mass index lower than other family members ³⁶. The risk of schizophrenia is higher in this population so regular monitoring is recommended ³.

This patient's parents had not yet been studied, so, we were not able to assess whether the duplication was inherited or *de novo*. However, the 16p11.2 duplication is frequently inherited, both from asymptomatic or affected parents. This CNV was classified as pathogenic.

CASE 20

The array-CGH performed on a 15-year-old male with ASD showed a Xp11.22 duplication with 1,2Kb. The duplication includes 10 genes: *HSD17B10*, *HUWE1*, *MIRLET7F2*, *PHF8*, *FAM120C*, *WNK3*, *FGD1*, *MIR98*, *TSR2* and *GNL3L*. This region overlaps the Xp11.22 microduplication syndrome (OMIM300705) and is responsible for mental retardation caused mainly by *HSD17B10* and *HUWE1*. The *HUWE1* is also considered responsible for neural differentiation and his over expression has been associated with the autistic phenotypes ³⁷.

An epigenetic regulation of *HUWE1* can be one of the mechanisms involved ³⁸. The parents were not yet available for study. This CNV was classified as pathogenic.

In the last few decades, the identification of genetic causes of autism increased considerably due to the widespread use of microarrays.

The array-CGH provides much more resolution than the karyotype, since it detects deletions or duplications much smaller than the 5Mb, the resolution of the karyotype (table 1). This supports the conclusion that array-CGH should be the first line of testing for developmental disorders ⁹.

In our data some carriers were *unaffected*, and this could be related with variable penetrance. In these cases, the penetrance may be incomplete, typically between 10% and 60% ³⁹. That can bring complexity to the genetic counselling and may result in the birth of affected children for an unsuspecting parent who passes his/her progeny a likely pathogenic CNV ⁷. Moreover, being informed that one carries the potential for an untreatable and in some cases adult-onset condition can also be stressful news.

There are some limitations associated with the technique: array-CGH cannot identify polyploidy or balanced rearrangements such as translocations and inversions. Nevertheless, this could have more impact in reproductive counselling for couples with infertility or spontaneous abortions or in the study of products from miscarriages. One of our additional purposes in this study was applying an NGS panel with the most frequent genes associated with autism in cases with a normal array result. This could improve detection rate of genetic alterations associated with the phenotype (table 3).

In fact, NGS could detect mutations in a specific sequence of genes coding for proteins that may be present in ASD children and absent in others ⁴⁰.

The NSG panel, namely those applied with at least 116 genes included can specially increase the detection rate variants. Moreover, most of the variants identified were classified as uncertain significance which may difficult the interpretation.

Conclusion

The results of our study are in accordance with the literature, highlighting the relevance of array-CGH as the first-tier genetic test in the ASD population. Our study reinforces the need for complementarity between array-CGH and NGS panels or whole exome sequencing in the etiological diagnosis of ASD. However, it is important to stress that at least for now we do not

recommend NGS panels as the first-tier diagnostic technique for the diagnosis of individuals with ASD but instead should be applied in the cases with array-CGH normal results and after the investigation of pathogenic variants of MECP2 gene in females and Fragile-X in males.

Sharing databases can help physicians improving diagnoses. The use of the same criteria of diagnosis (DSM-5) could help in the genotype-phenotype correlations. Physicians should continue to pay close attention to parental concerns and use validated assessment tools (eg, M-CHAT) to guide decisions about whether to continue the diagnostic evaluation and referral. Early referral to experts in ASD allows better outcomes for those children.

Management of results is important for families as the impact on genetic counselling is one of the points in need of deeper reflection. Interpreting data from carriers of CNVs is not always intuitive and can cause psychological harm.

In conclusion, more studies should be performed but we hope that present study has added some input in the genetic diagnosis of ASD patients in our hospital, either confirming or describing new CNVs related with ASD.

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Figure 1 – Flow chart of the CNVs classifications found in the 253 patients;

Legend: ASD – Autism spectrum disorders; CNVs – copy number variations; LP – likely pathogenic;

Table 1 - Pathogenic or likely pathogenic CNVs with association in ASD

Case	Sex	aCGH result	Cytoband	CNV type	Dimension (Kb)	Additional Findings	Additional exams and results	Clinical findings
1	M	arr 1p32.2(58,527,830-58,678,806)x1	1p32.2	LP	151 del	inherited from male progenitor	normal MLPA	
2	M	arr 1q21.1(146,506,310-147,824,207)x3	1q21.1	P	1318 dup	epilepsy		
3	M	arr 1q21.1q21.2(146,506,310-149,243,967)x3	1q21.1q21.2	P	2738dup	inherited from male progenitor		
4	M	arr2p11.2(86,294,476-86,513,002)x3	2p11.2	LP	219 dup	inherited from male progenitor	normal MLPA	
5	M	arr 2p25.3(1,742,241-1,848,126)x3	2p25.3	LP	106 dup			
		arr16p11.2(28,824,794-29,042,118)x3	16p11.2	LP	217 dup			
6	F	arr 3p26.3(73,914-320,575)x1	3p26.3	LP	247 del	inherited from male progenitor	normal MLPA, ish15q11-13(D15S10x2), NGS panel: a variant of uncertain pathogenicity: c.2981G>A (pArg994Gln) in the exon 3 of RAI1 gene	
7	M	arr 5p15.2(11,689,576-11,868,071)x1	5p15.2	LP	178del	inherited from male progenitor	normal karyotype, normal PAX6 gene	coloboma of the optic nerve, myopia
8	F	arr7p14.1(40,008,451-40,132,520)x3	7p14.1	LP	124dup		normal PCDH19 gene, normal MLPA	Diabetes <i>mellitus</i>
9	M	arr 7q11.22(69,242,038-69,269,099)x1	7q11.22	LP	27 del		normal MLPA, normal karyotype	
10	F	arr 9p24.2-p24.1(3,221,616-5,308,208)x1	9p24.2-p24.1	LP	2087 del			
11	F	arr 10q11.22 (49,390,457-52,415,071)x1	10q11.22	P	3025 del		normal MLPA, normal karyotype, normal ish subtel (41x2)	

12	M	arr 11q23.3(120,318,946-120,535,463)x1	11q23.3	LP	217 del	<i>de novo</i> alteration	normal karyotype, NGS panel of autism normal	Macrocephalic
13	M	arr15q11.2(22,765,628-23,208,901)x1	15q11.2	P	443 del		normal MLPA	
14	M	arr15q11.2(22,765,628-23,208,901)x1	15q11.2	P	443del	Inherited from female progenitor	normal MLPA	
15	M	arr 16p11.2(28,824,794-29,042,118)x3	16p11.2	P	217dup	microduplication syndrome chro. 16		Personality perturbation, low stature
16	M	arr 20p12.3 (7,591,735-8,656,221)x1	20p12.3	LP	1064 del		normal MLPA, normal karyotype	
17	M	arr 21q22.3(43,818,758-44,969,360)x3	21q22.3	LP	1151 dup		normal MLPA, normal karyotype, ish15q11-13(D15S10x2	
18	M	arr 21q22.3(46,811,103-47,121,128)x3	21q22.3	LP	310 dup			Macrocephalic
19	M	arr 22q11.21(18,894,835-19,165,016)x3	22q11.21	LP	270 dup		NGS panel of autism genes normal	
20	M	arr Xp11.22(53,459,179-54,672,305)x3	Xp11.22	P	1213dup		normal MLPA	
21	M	arr Xp22.31(6,489,877-8,131,810)x0	Xp22.31	P	1109 del		NGS panel of autism genes normal	
22	M	arr Xp22.33 (192,689-2,689,408)x2 or Yp11.32(142,689-2,689,408)x2	Xp22.33 + Yp11.32	P	2497 dup			

Legend: P – pathogenic; LP – Likely Pathogenic; del – deletion; dup – duplication;

Table 2 - CNVs without clear association with ASD

Case	Sex	aCGH result	Cytoband	CNV type	Dimension (Kb)	Additional Findings	Clinical findings
1	M	arr 6p25.3(266,079-328,956)x1,10q21.1 (56,185,390-57,236,526)x1	6p25.3 + 10q21.1	pathogenic in recessive forms	63 del + 1051 del	inherited from female progenitor	
2	M	arr 8p21.3(22,222,050-22,370,282)x3, arr21q22.3(46,811,103-47,121,128)x3	8p21.3 + 21q22.3	LB + LP	148dup + 310dup	inherited from female progenitor	
3	M	arr 2q33.3(207,639,004-207,657,132)x1	2q33.3	pathogenic in recessive forms	18del	inherited from male progenitor	epilepsy
4	M	arr 13q12.11-q12.12(22,995,810-23,907,017)x1	13q12.11-q12.12	pathogenic in recessive forms	911 del		
5	M	arr 1q21.1(146,542,843-146,628,179)x1, arr 14q11.2(23,167,693-23,302,619)x3, arr Yq11.222(20,805,776-21,071,104)x3	1q21.1 + 14q11.2 + Yq11.222	LP + LP + LP	85 del + 135 dup + 265 dup		
6	M	arr 22q11.21(18,909,044-19,147,457)x3	22q11.21	LP	238dup	<i>de novo</i> alteration	macrocephalic, obesity
7	M	arr Xp11.4 (41,586,982-42,464,126)x2	Xp11.4	LP	877dup	<i>de novo</i> alteration	
8	M	arr 1q21.1(145,388,355-145,833,054)x1, arr4q24q25(107,617,634-108,165,829)x3	1q21.1 + 4q24q25	LP + LP	444 del + 548 LP		
9	M	arr 19p13.2(12,615,605-12,814,116)x3	19p13.2	LP; MAN2B1 - pathogenic in recessive form	199 dup		
10	M	arr15q21.3 (58,812,999-58,860,957)x1	15q21.3	LP	48del		

Legend: P – pathogenic CNV; LP – Likely Pathogenic; LB – likely benign;

Table 3 - Results obtained with the NGS panel study in patients with a normal array-CGH result

Case	Sex	Age	Clinical indication	Panel	Result
1	M	5	ASD	autism spectrum Tier 2 - 62 genes included + XLMR panel	heterozygous mutation c.1624C> T (p.Q542X) in exon 18 of the FOXP1 gene
2	M	8	ASD	autism spectrum Tier 2- 62 genes included	heterozygous variant of uncertain significance c.4981C> T (p.R1661W) in the RAI1 gene.
3	F	2	ASD	autism spectrum Tier 2 – 62 genes included	normal
4	F	8	ASD	Fulgent Autism panel of 45 genes	3 heterozygous variants of uncertain significance in the genes <i>CNTNAP5</i> , <i>GNA14</i> and <i>SMG6</i> .
5	F	7	ASD and epilepsy	Epilepsy panel EPI02: Epileptic Encephalopathy, version 6.1	normal
6	M	5	ASD, family history of schizophrenia	Fulgent Autism panel of 116 genes	normal
7	M	15	ASD	Fulgent Autism panel of 117 genes + XLMR memory panel	Heterozygous variants of uncertain significance in the <i>ANKRD11</i> , <i>NIPBL</i> and <i>SMG6</i> genes
8	F	2	ASD and obesity	Fulgent Autism panel of 117 genes	Heterozygous variants of uncertain significance in the <i>PDCH19</i> and <i>SYNE1</i> genes.
9	F	7	ASD	Fulgent Autism panel of 116 genes	heterozygous variants of uncertain significance in the <i>AMT</i> and <i>DOCK4</i> genes.
10	M	2	ASD	Fulgent Autism panel of 117 genes	heterozygous variants of uncertain significance in the <i>SLC2A1</i> , <i>SLC6A4</i> , <i>CHD7</i> and <i>SYNE1</i> genes
11	M	9	ASD	Fulgent Autism panel of 118 genes	heterozygous variant of uncertain significance in the <i>SHANK3</i> gene.
12	F	9	ASD	autism spectrum Tier 2 – 62 genes included	normal
13	M	2	ASD and mental retardation	Painel Fulgent XLMR + Fulgent Autism panel of 116 genes	heterozygous variants of uncertain significance in the <i>SCN2A</i> and <i>ST7</i> genes
14	M	5	ASD	Fulgent Autism Panel of 122 genes	normal

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Anexos

1. Normas da Revista – Neuropediatrics - *Journal of Pediatric Neurobiology, Neurology and Neurogenetics*
2. Parecer da Comissão de Ética para a Saúde do Centro Hospitalar São João

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- ☐ The preferred format for video submissions is MPEG-1.
- ☐ Please include a descriptive legend at the end of your main document, which will be published together with a link to your video.
- ☐ All video will be subject to peer review. They should be up to 2 minutes in length or a maximum of 10MB, appropriately labeled with a voiceover. QuickTime or AVI formats are acceptable. Authors who want their videos accessible in a streaming format must also provide either a single Sure-Stream file or 3 uniquely named single-rate clips (28.8, 56, T1) with a SMIL file to list the bandwidth choices. Video clips must meet production quality standards without modifications or editing by the Editorial Office. The Journal can accept only video submissions that meet the Journal's formatting and image quality requirements. Authors will be notified if there are any problems with submitted files and asked to resubmit modified files. Image editing and correct formatting are the author's responsibility.

VIDEOS AND IMAGES IN NEUROPEDIATRICS

General Guidelines

We encourage the submission of videos or images that exemplify specific aspects or symptoms of common or rare neurologic diseases in childhood. Emphasis should be laid on clinical signs that have a high recognition factor and that cannot easily be identified from written description only. “Images” may also include results of imaging procedures like MRI, CT, radiographs or ultrasound that depict instructive examples of recognizable patterns.

Formal requirements are: no abstract, word count max. 200, max. 1 video or 3 images, max. 1 table, and max. 5 references.

An informed consent letter signed by parents and children (if applicable) is a prerequisite. This does not account for anonymized MRI, CT, ultrasound, or radiographs. Besides originality, there are no restrictions on content or type of graphic presentation. This format undergoes a formal review procedure and is accounted for as a scientific contribution.

Videos should be up to 5 minutes in length. QuickTime or AVI formats are acceptable. Authors who want their videos accessible in a streaming format must also provide either a single Sure-Streamfile or 3 uniquely named single-rate clips (28.8, 56, T1) with a SMIL file to list the bandwidth choices. Video clips must meet production quality standards without modifications or editing by the Editorial Office. Authors will be notified if there are any problems with submitted files and asked to resubmit modified files. Each segment should be appropriately labeled and have transitions between video clips.

Authorship

We believe it important to document the adequate participation of all authors. We request no more than 6 authors be included. In all cases, however, multiple contributions of each author must be documented in our required form addressing copyright transfer, authorship, and conflicts of interest.

Conflict of Interest

All potential conflicts of interest must be declared by all named authors. This information shall be published in the accepted manuscript.

Title Page

The title page must include the following:

- ☐ Title (containing fewer than 80 characters including spaces)
- ☐ Running title (containing fewer than 40 characters including spaces)
- ☐ Author name(s) and final degree(s)
- ☐ The affiliation(s), and address(es), and e-mail addresses of all author(s)
- ☐ A statement of the location where the work was performed (only if authors from multiple institutions)
- ☐ Word Count: Limit manuscript to max. 200 words
- ☐ The Corresponding Author name and e-mail address (must be same as Corresponding Author in Manuscript Central)

DIGITAL ARTWORK PREPARATION

General Guidelines

- ☐ It is best to use Adobe Photoshop to create and save images, and Adobe Illustrator for line art and labels.
- ☐ Do not submit art created in Microsoft Excel, Word, or PowerPoint. These files cannot be used by the typesetter.
- ☐ Save each figure in a separate file.
- ☐ Do not compress files.
- ☐ All black-and-white and color artwork should be at a resolution of 300 dpi (dots per inch) in TIFF format. Line art should be 1,200 dpi in EPS or TIFF format. Contact the Production Editor at Thieme if you are unsure of the final size.
- ☐ It is preferable for figures to be cropped to their final size (approximately 3½ inches for a single column and up to 7 inches for a double column), or larger, and in the correct orientation. If art is submitted smaller and then has to be enlarged, its resolution (dpi) and clarity will decrease.

Note: Lower resolutions (less than 300 dpi) and JPEG format (.jpg extension) for grayscale and color artwork are strongly discouraged due to the poor quality they yield in printing, which requires 300 dpi resolution for sharp, clear, detailed images. JPEG format, by definition, is a lower resolution (compressed) format designed for quick upload on computer screens.

Black-and-White Art

- ☐ Black-and-white artwork can be halftone (or grayscale) photographs, radiographs, drawings, line art, graphs, and flowcharts. Thieme will only accept digital artwork.
- ☐ If possible, do not send color art for conversion to black-and-white. Do the conversion yourself so that you can check the results and confirm in advance that no critical details are lost or obscured by the change to black-and-white.
- ☐ For best results, line art should be black on a white background. Lines and type should be clean and evenly dark. Avoid screens or cross-hatching, as they can darken or be uneven in printing and lead to unacceptable printing quality.

Color Art

- ☐ Color illustrations are expensive to produce and usually cannot be accepted unless the author is willing to cover the additional production costs incurred. Please check with the Editor in Chief or Thieme for details. We will convert color illustrations to black-and-white unless we receive a letter from the author assuming responsibility for the cost of printing color. Upon request, we will provide you with a cost estimate for the color printing.
- ☐ All color artwork should be saved in CMYK, not RGB.

Art Labels

- ☐ Arrows, asterisks, and arrowheads (or other markers) should be white in dark or black areas and black in light or white areas, and large in size. If not, these highlighting marks may become difficult to see when figures are reduced in size during the typesetting process.
- ☐ Use 1-point (or thicker) rules and leader lines.
- ☐ Capitalize the first word of each label and all proper nouns. Consider using all capitals if you need a higher level of labels.
- ☐ Where there are alternate terms or spellings for a named structure, use the most common one and make sure it is consistent with what is used in the text.
- ☐ Avoid using multiple fonts and font sizes for the labels; use only one or two sizes of a serif font.

Parecer da Comissão de Ética para a Saúde do
Centro Hospitalar de São João / Faculdade de Medicina da Universidade do Porto

Título do Projeto: Avaliação citogenética por array-CGH em doentes com perturbações do espectro autista

Nome da Investigadora Principal: Sidonie Irene Lourenço Monteiro, estudante do MIMED da FMUP

Onde decorre o Estudo: No Serviço de Genética da FMUP. Dispõe de autorização do Prof. Doutor Alberto Barros. O profissional de ligação será o Dr. Miguel Leão, que é também co-orientador.

Objectivos do Estudo:

Estudo da prevalência e do tipo de alterações citogenéticas (CNVs) encontradas nas amostras de sangue periférico com indicação para estudo por array-CGH em doentes com perturbações do espectro autista, no Hospital de São João, de Abril de 2012 a Junho de 2017, bem como de alguns pais, quando a alteração citogenética indica o estudo dos progenitores.

Inserir-se no âmbito do Mestrado Integrado em Medicina da FMUP, sob orientação da Prof.^a Doutora Sofia Dória.

Concepção e Pertinência do estudo:

As perturbações do espectro autista afectam cerca de 1% da população, estando preconizado que deve ser realizado um estudo genético, incluindo um cariótipo e uma análise por hibridação genómica comparativa em arrays (aCGH).

Benefício/risco: Não aplicável

Confidencialidade dos dados:

Visando assegurar o sigilo dos dados individuais, será criado um número de código identificativo, cuja descodificação será exclusivamente acessível ao médico assistente e ao investigador.

Respeito pela liberdade e autonomia do sujeito de ensaio: Não aplicável

Curriculum da investigadora: Adequado à investigação.

Data previsível da conclusão do estudo: Fevereiro de 2019

Conclusão: Proponho um parecer favorável à realização deste projecto de investigação.

Porto, 15 de Junho de 2018

O Relator da CES, Prof. Doutor Filipe Almeida

Questionário para submissão de Investigação

Exmo. Sr. Presidente da Comissão de Ética do Centro Hospitalar de São João/
Faculdade de Medicina da Universidade do Porto,

Pretendendo realizar a investigação infracitada, solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer. Para o efeito, anexo toda a documentação requerida.

IDENTIFICAÇÃO DO ESTUDO

Título da investigação: Avaliação citogenética por array-CGH em doentes com perturbações do espectro autista

Nome do investigador: Sidonie Irene Lourenço Monteiro

Endereço eletrónico: sidoniemonteiro22@gmail.com

Contacto telefónico: 918549268

Caracterização da investigação:

☒ Estudo retrospectivo

☐ Estudo observacional

☐ Estudo prospetivo

☐ Inquérito

☐ Outro. Qual? _____

Tipo de investigação:

☒ Com intervenção

☐ Sem intervenção

Formação do investigador em boas práticas clínicas (GCP): ☒ Sim ☐ Não

Promotor (se aplicável): _____

Nome do orientador de dissertação/tese (se aplicável): Sofia Dória Príncipe dos Santos Cerveira

Endereço eletrónico: sdoria@med.up.pt

Local/locais onde se realiza a investigação: Departamento de Genética – FMUP

Data prevista para início: 19 / 02 / 2018

Data prevista para o término: 01 / 02 / 2019

PROTOCOLO DO ESTUDO

Síntese dos objetivos:

Estudo da prevalência e do tipo de alterações citogenéticas (CNVs) encontradas nas amostras de sangue periférico com indicação para estudo por array-CGH em crianças com perturbações do espectro autista, no Hospital de São João, de abril de 2012 a junho de 2017, bem como de alguns pais quando a alteração citogenética indica o estudo dos progenitores.

Fundamentação ética (ganhos em conhecimento/inação; ponderação benefícios/riscos):

As perturbações do espectro autista afetam cerca de 1% da população, estando preconizado que deve ser realizado um estudo genético, incluindo um cariótipo e uma análise por hibridação genómica comparativa em arrays (aCGH). Aquando da análise por aCGH, existem variações de significado indeterminado, classe IIIa, que devem ser alvo de discussão com base a base de dados criada.

Devem ser tidas em conta as alterações de classe I e II e de como a maioria dos autores defende o estudo dos progenitores, tendo isso não sido feito até a data.

A maioria dos autores defende o estudo dos progenitores quando uma criança do sexo masculino tem alteração do cromossoma X e com a mãe assintomática, permitindo estudar um fenómeno de inativação de cromossoma X e assim apoiar a preponderância pela afetação do sexo masculino (ratio de 4:1).

Poder-se-á também avaliar o aconselhamento genético futuro dessas famílias estudadas.

Não se preveem riscos relevantes.

CONFIDENCIALIDADE

De que forma é garantida a anonimização dos dados recolhidos de toda a informação?

A todos os pacientes é garantido o sigilo dos dados individuais. Com esse objetivo será criado um número de código identificativo, cuja decodificação será única e exclusivamente do conhecimento do médico assistente e do investigador do estudo.

O investigador necessita ter acesso a dados do processo clínico?

☒ Sim ☐ Não

Está previsto o registo de imagem ou som dos participantes?

☐ Sim ☒ Não

Se sim, está prevista a destruição deste registo após o sua utilização?

☐ Sim ☐ Não

CONSENTIMENTO

O estudo implica recrutamento de:

Doentes: ☐ Sim ☒ Não

Voluntários saudáveis: ☐ Sim ☒ Não

Menores de 18 anos: ☐ Sim ☒ Não

Outras pessoas sem capacidade do exercício de autonomia:

☐ Sim ☒ Não

A investigação prevê a obtenção de Consentimento Informado:

☐ Sim ☒ Não

Se não, referir qual o fundamento para a isenção:

O consentimento já foi pedido para a realização do estudo genético

Existe informação escrita aos participantes: ☐ Sim ☒ Não

PROPRIEDADE DOS DADOS

A investigação e os seus resultados são propriedade intelectual de:

☐ Investigador ☐ Promotor ☐ Ambos ☒ Serviço onde é realizado

☐ Não aplicável

Outro: _____

BENEFÍCIOS, RISCOS E CONTRAPARTIDAS PARA OS PARTICIPANTES

Benefícios previsíveis:

Identificação de hereditariedade familiar de perturbações do espectro autista.

Riscos/incómodos previsíveis:

Deslocação ao hospital para recolha de sangue periférico.

São dadas contrapartidas aos participantes:

· pela participação ☐ Sim ☐ Não ☒ Não aplicável

· pelas deslocações ☐ Sim ☐ Não ☒ Não aplicável

· pelas faltas ao emprego ☐ Sim ☐ Não ☒ Não aplicável

· por outras perdas e danos ☐ Sim ☐ Não ☒ Não aplicável

CUSTOS / PLANO FINANCEIRO

Os custos da investigação são suportados por:

☐ Investigador ☐ Promotor ☒ Serviço onde é realizado

☐ Não aplicável

Outro: _____

Existe protocolo financeiro? ☐ Sim ☐ Não

LISTA DE DOCUMENTOS ANEXOS

- ☐ Pedido de autorização ao Presidente do Conselho de Administração do Centro Hospitalar de São João (se aplicável)
- ☐ Pedido de autorização à Diretora da Faculdade de Medicina da Universidade do Porto (se aplicável)
- ☒ Protocolo do estudo
- ☒ Declaração do Diretor de Serviço onde decorre o estudo
(sendo um estudo na área de enfermagem deve anexar também a concordância da chefia de enfermagem)
- ☒ Profissional de ligação
- ☒ Informação dos orientadores
- ☐ Informação ao participante
- ☐ Modelo de consentimento
- ☐ Instrumentos a utilizar (inquéritos, questionários, escalas, p.ex.): _____
- ☒ Curriculum Vitae abreviado (máx. 3 páginas)
- ☐ Protocolo financeiro
- ☐ Outros:

COMPROMISSO DE HONRA E DECLARAÇÃO DE INTERESSES

Declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (1960 e respetivas emendas), e da Organização Mundial da Saúde, Convenção de Oviedo e das "Boas Práticas Clínicas" (GCP/ICH) no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo, nos últimos três meses. Comprometo-me a entregar à CES o relatório final da investigação, assim que concluído.

Porto, 24 de Maio de 2018

Nome legível: Sidanie Irene Lourenço Monteiro

Sidanie Irene Lourenço Monteiro
assinatura

Parecer da Comissão de Ética do Centro Hospitalar de São João/FMUP

Emitido na reunião plenária da CE de 5 / 06 / 18

A Comissão de Ética para a Saúde
APROVA por unanimidade o parecer do
Relator, pelo que nada tem a opor à
realização deste projecto de investigação.

Filipe Almeida
Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética