

Etiological Investigation of Autism Spectrum Disorders – State of The Art

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ABSTRACT

Autism Spectrum Disorder is a Neurodevelopmental Disorder characterized by deficits in social interaction and by the presence of restricted, repetitive and stereotyped patterns of behaviours, interests, and activities. The aetiology of Autism Spectrum Disorder is often genetic, with several monogenic diseases clearly associated with this disorder. Significant advances in molecular genetics have increased the rate of etiological diagnosis of Autism Spectrum Disorder to about 30-40% in the last decade.

The establishment of a definitive etiological diagnosis facilitates referral to community support services, contributes to knowledge of possible associated medical conditions and prevention of morbidity and mortality, while also eliminating inadequate diagnostic tests and allowing individualized genetic counselling.

The authors present a proposal for an etiological investigation of this pathology, including criteria for performing complementary metabolic evaluation, neuroimaging and electroencephalography, and various genetic studies (conventional cytogenetics, Array-Comparative Genomic Hybridization, targeted molecular studies, multi-gene panels and Whole Exome Sequencing).

Keywords: Autism; genetics; neurodevelopment

INVESTIGAÇÃO ETIOLÓGICA DA PERTURBAÇÃO DO ESPETRO DO AUTISMO – O ESTADO DA ARTE

RESUMO

A Perturbação do Espectro do Autismo é uma Perturbação do Neurodesenvolvimento, que se caracteriza por défice na interação social e pela presença de padrões restritos, repetitivos e estereotipados de comportamentos, interesses e atividades. A etiologia das Perturbações do Espectro do Autismo é frequentemente genética, existindo várias doenças monogénicas claramente associadas a esta perturbação. Avanços significativos na genética molecular aumentaram a taxa de diagnóstico etiológico para cerca de 30 a 40% na última década.

O estabelecimento de um diagnóstico etiológico definitivo facilita a referenciação para os serviços de apoio na comunidade, contribui para o conhecimento de eventuais condições médicas associadas e para a prevenção da morbimortalidade, elimina a realização de exames auxiliares de diagnóstico inadequados e facilita o aconselhamento genético individualizado.

Os autores apresentam uma proposta de investigação etiológica desta patologia, incluindo critérios para realização de avaliação metabólica complementar, realização de neuroimagem e eletroencefalograma e variados estudos genéticos (citogenética convencional, arrays de hibridização genómica comparativa, estudos moleculares dirigidos, painéis multigénicos e sequenciação exómica completa).

Palavras-chave: Autismo; genética; neurodesenvolvimento

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a Neurodevelopmental Disorder characterized by persistent deficits in social communication and social interaction across multiple contexts, and by the presence of restricted, repetitive and stereotyped patterns of behaviours, interests or activities.¹ ASD phenotype is extremely heterogeneous, with great individual variability, both of displayed signs and symptoms and in severity.

Incidence of ASD appears to be increasing in the last two decades, although it's not clear whether this results from a real increase in incidence, modification of diagnostic criteria, increasing knowledge by healthcare professionals or a combination of these factors.^{2,3}

In the largest American study, cited in several literature reviews, the estimated prevalence at eight years was 14.7 in 1000 children (1 out of 68), with a clear predominance of males (1 in 42 vs. 1 in 189 in females).⁴ In Portugal, in a prevalence study carried out in 1999, the prevalence of ASD was much lower than previously reported, and was estimated at only 0.92 in 1000 children.⁵

Considering the high prevalence of this pathology and the affection of the quality of life of these children and their families, etiological investigation becomes fundamental. A definitive etiological diagnosis allows referral to specific support services, can contribute to knowledge of possible associated pathologies, to prevent morbidity and to define the prognosis, and is also essential for individualized genetic counselling, estimation of the risk of recurrence specific to each family and informed planning of reproductive options. Significant advances in genetic research have increased the diagnostic rate from 6-10% in the first decade of this century, to 30-40% in some recent series, as evidenced by the recommendations of the *American College of Medical Genetics* in 2013.⁶

Growing evidence supports the pivotal role of genetics in the aetiology of ASD, and thus genetic research should be offered to all families of patients with ASD.⁷ The purpose of this review is to provide a comprehensive evaluation of the current evidence regarding the usefulness of major diagnostic tests clinically available (genetical, biochemical, imaging) in order to achieve etiological diagnosis in a patient with ASD. A thorough search for relevant studies was performed in MEDLINE and PUBMED, with the following MeSH words: aCGH, ASD, autism, brain, CNV, cytogenetics, dysmorphism, electroencephalogram, epilepsy, etiology, exome, FMR1, genetics, karyotype, MECP2, metabolic, MRI, neuroimaging, panel, PTEN, seizures, sequencing, syndromic and WES. Major available clinical guidelines were also consulted.^{6,8} We did not restrict by year of publication or publication status.

THE ROAD IN THE ETIOLOGICAL INVESTIGATION OF ASD

Essential autism vs. complex or syndromic autism

Distinction between essential (idiopathic) and complex (syndromic) autism is usually the first step in the etiological investigation of ASD.⁹ Complex forms of autism are characterized by evidence of an abnormality in early morphogenesis, manifested by either dysmorphic signs or cutaneous lesions on physical

examination, microcephaly or associated congenital malformations. Examples of syndromic autism include Tuberous Sclerosis, Fragile-X Syndrome, Angelman, CHARGE, Coffin-Lowry, Cohen, de Lange, Down, Moebius, Phelan-McDermid, Prader-Willi, Rett, Sanfilippo, Smith-Lemli-Opitz, Smith-Magenis, Sotos, Timothy and 22q11.21 deletion syndromes. Clinical recognition of monogenic diseases clearly associated with ASD allows for a targeted diagnostic approach. It should be clearly stated that syndromic forms of ASD are not simply ASDs whose genetic causes are known, but are different clinical entities with different developmental trajectories from nonsyndromic ASD.¹⁰ Higher rates of diagnostic accuracy are reported in children with syndromic or complex ASD, and performing some investigations like neuroimaging are dependent on this syndromic vs. idiopathic differentiation.

However, most cases of ASD (about 75%) are forms of essential autism, in which the absence of particular neurological or morphological signs doesn't suggest a specific diagnosis (genetic or otherwise), and therefore doesn't allow for targeted molecular studies.¹⁰

Taking into account the above, it is fundamental to collect a detailed clinical history and to perform an adequate physical examination, with particular relevance to growth parameters, dysmorphisms, skin and neurological examination.

Metabolic investigation

There are several metabolic diseases already identified in children with ASD, although these are relatively rare and usually have an early clinical presentation. The two most common metabolic diseases associated with ASD are brain deficiencies of folate (5-methyltetrahydrofolate) and mitochondrial disorders.¹¹ Most international recommendations do not advocate for a routine extensive metabolic workup, taking into account the low yield of diagnosis and inherent costs.⁶ However, a high index of suspicion is necessary and, when in the setting of certain clinical and analytical findings associated with metabolic diseases, a metabolic workup should be carried out. Presence of failure to thrive, microcephaly, coarse facial features, recurrent disorders of consciousness, paroxysmal disorders of movement (epilepsy, dystonia), cognitive deterioration (especially if after infections or immunizations), hearing or visual impairment, recurrent hypoglycaemia, recurrent episodes of vomiting and dehydration, anaemia with elevated mean corpuscular volume, acid-base or electrolyte disturbances, unusual odours, multi-system involvement (specially cardiac, hepatic or renal) and dermatological changes (alopecia, hypertrichosis, pigmented rash), increase the odds of a metabolic disease, requiring complementary metabolic investigation.⁶

Central nervous system imaging

The role of neuroimaging in the etiological investigation of ASD or other neurodevelopmental disorders has been questioned in recent years. Although the rate of detected abnormalities is high (up to 48% in some studies), most of them are not diagnostic of any clinical entity.^{12,13} Therefore, current evidence only suggests performing a brain MRI in children with ASD with concomitant

macrocephaly (above p98), microcephaly, marked cognitive regression, epilepsy, neurocutaneous disorders or dysmorphic features on physical examination.⁶

Electroencephalography

The electroencephalogram (EEG) it's not routinely recommended in the workup investigation of ASD. Although the presence of epileptiform activity is a frequent electroencephalographic finding in children with ASD, without clinical criteria of epilepsy, it's not clear the relationship of these anomalies with the clinical manifestations characteristic of ASD.¹⁴ It is recommended to perform an EEG (including sleep record) in the setting of epilepsy or when the patient has a cognitive regression, mainly to exclude an Electrical Status Epilepticus in Sleep (ESES), a clinical entity associated with regression of language (with some similarities to the ASD-associated regression), or regression of other neurodevelopment areas.¹⁵

The major role of genetics

There is now a large scientific evidence to support the role of pathogenic genetic variants in the aetiology of ASD.¹⁶ Due to an ever-growing number of genes clearly associated with ASD, genetic research should always be carried out, taking into account variables such as the usefulness of the diagnosis for that particular child (including possible treatment opportunities), family genetic counselling (including defining recurrence risk and planning of reproductive options), the sensitivity, specificity, and the cost of the tests to be performed. The reported success rate in identifying the aetiology of ASD is variable, being very influenced by the clinical expertise of the observer, the type of patients studied and the techniques used. In some international studies, the diagnostic yield reaches 25%.^{17,18}

Conventional cytogenetics

There are numerous deletions or duplications detectable in conventional cytogenetic studies (conventional karyotype) associated with ASD.¹⁹ However, the advent of molecular cytogenetic techniques such as the *Array-Comparative Genomic Hybridization (aCGH)* has rapidly replaced conventional cytogenetics and is now considered the state-of-the-art first-line genetic test in the investigation of ASD.²⁰ Therefore, conventional cytogenetic techniques are only recommended in the clinical suspicion of aneuploidy or if the family or reproductive history is suggestive of chromosomal rearrangements (infertility or repetitive abortion).

FMR1 gene mutations

There is a clear association between Fragile-X Syndrome and ASD in males. Approximately 30-50% of males with Fragile-X Syndrome meet full ASD criteria, and the estimated incidence of Fragile-X Syndrome in children with ASD has been reported in the range of 0.5-5%.^{21,22} It is recommended that all ASD males who also meet criteria for an Intellectual Disability Disorder should be tested for the usual expansion of the CGG triplet in the FMR1 gene, which is responsible for most cases of this disease. Regarding females, there is no scientific evidence

to support routine search for FMR1 mutation, and thus it is only recommended if there is a family history suggestive of X-Linked Developmental Disorders, premature ovarian failure, or tremor/ataxia syndrome, clinical manifestations frequently associated with premutation carriers for Fragile-X syndrome.⁶

Array-Comparative Genomic Hybridization (aCGH)

This is a molecular cytogenetic technique that analyzes the presence of Copy Number Variations (CNVs) - deletions and duplications - relative to a "reference genome". Since this technique only detects variations in the number of copies, it does not detect reciprocal translocations, inversions or ring chromosomes, changes that do not affect the number of copies.²³ According to more recent studies, approximately 10% of children diagnosed with ASD have pathogenic CNVs identified by aCGH.¹⁷ In some series, restricting this test to children with comorbidities (epilepsy or various types of congenital anomalies), raises the diagnostic yield to about 30%.²⁴ The introduction of aCGH into routine clinical services has been slow due to the perceived high cost of the test and because of the long established acceptance in routine clinical practice of karyotyping as the first-line test for either ASD and Intellectual Disability. Cost-effectiveness studies of using aCGH as a first-line tier for major developmental disorders conclude that it's cost saving, limiting the use of additional tests.²⁵ Since 2010, the majority of revised international guidelines for ASD investigation propose aCGH as the first-tier clinical diagnostic test.^{6,26} To our knowledge, aCGH testing should be offered to all children with ASD when, after a detailed medical history and physical examination, the clinician doesn't have any clues to suggest a specific cause of ASD.

MECP2 Sequencing

Rett Syndrome is a severe neurodevelopmental disorder, usually caused by mutations in the MECP2 gene, located on X chromosome.²⁷ Several years after the discovery of this mutation, it became clear that this gene is implicated in many other phenotypes besides Rett Syndrome, one of which is the phenotype of idiopathic autism.²⁸ Up to 4% of females with idiopathic autism have pathogenic mutations in the MECP2 gene, presenting with a phenotype similar to the Rett Syndrome variant without microcephaly and with preservation of speech.²⁹⁻³¹ Therefore, MECP2 sequencing is recommended in all female individuals with idiopathic autism. Regarding males, MECP2 routine sequencing is not recommended, but only if the phenotype is compatible with MECP2 duplication syndrome, such as moderate to severe intellectual disability, sialorrhea, childhood hypotonia, epilepsy, and recurrent respiratory infections.

PTEN associated diseases

Germline mutations in the PTEN gene are described in a variety of rare syndromes known collectively as PTEN-Hamartoma-Tumor Syndromes (PHTS), of which the most commonly described in medical literature is Cowden Syndrome.

The presence of multiple hamartomas and tumour susceptibility at various sites are usually the most distinctive features of PHTS.³² However, one of the phenotypes clearly associated with PTEN mutations is one that includes autism and macrocephaly, without hamartomas or other signs of PTEN-associated disease.³³ Up to 5% of children with clinical criteria for ASD and macrocephaly (above age-adjusted p98) have mutations in the PTEN gene.³⁴ It is therefore recommended to search for PTEN mutations in all children with ASD and macrocephaly above p98.

Multi-gene panels, whole exome sequencing and whole genome sequencing

Although the first Mendelian mutation was identified in the 1980s, less than 200 Mendelian genes were known by the year 2000.³⁵ With the publication of the human genome in 2001, thousands of genes have been identified as disease-causing (15750 as of October 2017).^{36,37} In order to achieve the benefits of this growing knowledge of genes that cause human diseases, genome sequencing tools have quickly been introduced in clinical practice (particularly Next-Generation Sequencing). While sequencing of the entire genome (coding and non-coding regions) is feasible, it is still only investigational, and its application in the etiological investigation of ASD is not presently indicated.³⁸ Thus, sequencing of its 2% coding part (Whole Exome Sequencing - WES) has emerged as a cheaper and more practical alternative.³⁹ Recently WES has been extensively applied in studies of several pathologies, allowing for identification of multiple genetic variants implicated in ASD.⁴⁰ A few large studies on the clinical utility of WES on a range of disorders (mostly neurological) have reported a yield of around one in four, making it the highest yield test clinically available at the time of this review.⁴¹ But the financial cost of clinical-grade WES is high and WES typically uncovers numerous variants, so identifying the one causal variant can be a challenge.⁴² To overcome the drawbacks of WES multi-gene panels (in which an assortment of genes deemed relevant to a particular phenotype are sequenced), are more feasible, cheaper and provide faster results. Multi-gene ASD panels (in which frequently more than 100 genes clearly associated with ASD are sequenced), are rapidly becoming a key clinical instrument in the etiological investigation of ASD.⁴³ When family history is consistent with an X-linked pattern of inheritance and the patient, in addition to the ASD, and fulfils criteria for an Intellectual Disability Disorder, a multi-gene panel for X-linked Intellectual Disability should also be considered, because there are several X-linked genes associated both with ASD and Intellectual Disability.⁴⁴ We recommend performing multi-gene panels for either ASD and X-Linked Intellectual Disability or WES in a specific medical genetics consultation, to be defined individually and taking into account the reproductive expectations of the family.

CONCLUSION

Autism Spectrum Disorder is a serious neurodevelopmental disorder that involves a complex interaction between genetic

factors, with an ever-growing number of genes involved, and environmental factors. A large variability of rare chromosomal abnormalities, copy number variations, and point mutations account for a significant percentage of ASD. Genetic testing has rapidly progressed in recent years and has already become incorporated into daily routine clinical practice. It is very important that paediatricians have a basic understanding of the range of possible tests, indications for their utility and pitfalls in their interpretation. The application of genetic testing for common disorders is expanding each day, and such tests will likely be extended from diagnosis to assess the susceptibility to common multifactorial disorders and to predict the response to a specific medication. However, the contribution of the clinical history and medical examination of the child remains fundamental in the detection of dysmorphic signs or other findings that may be determinant for a targeted approach. According to what is currently believed to be “state-of-the-art” medical knowledge, we suggest a sequential approach in the etiological investigation of Autism Spectrum Disorder, which, like all clinical investigations, should be contextualized and directed to each individual patient and family, and should not be considered as a universally applicable algorithm to all patients with ASD.

Sequential approach to establish etiology of ASD

Detailed clinical history, family history and physical examination, along with confirmation of the diagnosis of ASD; rule out hearing loss has a contributing factor to communication and behaviour difficulties.

1. Sequential evaluation

- a. Targeted molecular study if any clinical suspicion*
- b. EEG in the presence of epilepsy or if cognitive deterioration**
- c. Metabolic study if any signs or symptoms suggestive of metabolic disease***
- d. FMR1 testing (Fragile-X Syndrome) in all males with Intellectual Disability Disorder
 - i. FMR1 testing in females if family history of X-linked neurodevelopmental disorders, premature ovarian failure, tremor/ataxia syndrome
- e. aCGH (Array-Comparative Genomic Hybridization)
- f. Conventional karyotype if family history suggestive of chromosomal rearrangements (infertility, repeat abortions)
- g. MECP2 sequencing in all females
 - i. MECP2 sequencing in males if sialorrhea, recurrent respiratory infections, hypotonia and dystonia
- h. Brain MRI if macrocephaly above p98, microcephaly, regression, epilepsy or dysmorphic features
- i. PTEN sequencing if macrocephaly above p98
- j. Multi-gene panels for ASD and X-Linked intellectual disability or Whole Exome Sequencing, to be defined individually in a Medical Genetics consultation, taking into account the reproductive expectations of the family

* Syndromes with frequent association with ASD: Angelman, CHARGE, Coffin-Lowry, Cohen, Cornelia de Lange, Down, DiGeorge, Tuberous Sclerosis, Moebius, Phelan-McDermid, Prader-Willi, Rett, Sanfilippo, Smith-Lemli-Opitz, Smith-Magenis, Sotos, Timothy, Fragile-X, PTEN-Hamartoma-Tumor and 22q11.21 deletion syndromes.

** Regression / cognitive deterioration beyond the “typical” language regression between 18-24 months

*** In the setting of: failure to thrive, microcephaly, coarse facial features, recurrent disturbances of consciousness, paroxysmal movement disorders (epilepsy, dystonia), cognitive deterioration (especially if after infections and immunizations), hearing or visual impairment, recurrent hypoglycaemia, recurrent episodes of vomiting and dehydration, anaemia with high mean corpuscular volume, acid-base or electrolyte disturbance, multisystemic involvement (specially cardiac, hepatic or renal), skin problems (alopecia, hypertrichosis, pigmented rash).

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