

Autism Spectrum Disorders: An Intervention Approach Based on Genomic Analysis

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Neurodevelopmental disorders are mainly represented by autism spectrum disorders (ASD) commonly known as autism, attention deficit hyperactivity disorder (ADHD), cerebral palsy, learning disabilities, developmental delays and intellectual retardation. A worrying situation is that neurodevelopmental disorders have become increasingly frequent over the past 30 years worldwide. In fact, the Autism Society of America has recently reported that autism is the fastest growing developmental disability, increasing annually at a rate of 10 to 17 percent.

At least in part, this trend is explained by the increase of environmental pollution and inadequate nutritional balance at early stages of pregnancy, because the developing brain is extremely vulnerable to these factors. Moreover, if coexist genetically determined innate vulnerabilities these environmental agents could be act as extremely harmful factors even at doses much lower than those that affect people without special genetic vulnerabilities.

In the present paper I describe a method that, due to a particular genetic predisposition, makes it possible to understand how external factors acting together with internal factors, might induce various gene-environment interactions with differential impact on the clinical outcome in ASD patients. This method is based on previously published papers and preliminary results of a study carried out in our center.

Thus, the aim of the present paper is to insist in the need of including genetic characterization of vulnerabilities to environmental influences as part of the analysis protocol for all ASD patients. By applying this method we propose an approach that does not represent a DNA test to confirm a diagnosis, but a DNA-SNP analysis to detect especial vulnerabilities to several environmental factors in a multifactorial context. In this sense, I propose a method based on DNA polymorphism analysis as the first step to characterize vulnerabilities related to different ASD subtypes in order to design more individualized therapeutic and support strategies.

Keywords: Autism Spectrum Disorders (ASD); Attention Deficit Hyperactivity Disorder (ADHD); Genetics; DNA polymorphism; Gene-environment interactions; Autistic Disorder/metabolism; Autistic Disorder/pathology; Autistic Disorder/subtypes; DNA methylation; Epigenetics

Introduction

Clinical results of neuroimaging, pathological and neurochemical studies have shown that autism spectrum disorders (ASD) are diseases of neuronal-cortical organization that cause deficits in information processing in the nervous system, from the synaptic and dendritic organization to connectivity and brain structure. These changes probably alter the development of social and communication path and seem to be affected by genetic and environmental factors.

In spite that previous studies reported an estimate of heritability of Autism at about 90% [1], the most recent study carried out at the University of California reported a lower rate of heritability of 37% and 38% for autism and ASD, respectively [2].

Notwithstanding of a data discrepancy in measuring the contribution of gene and environment to ASD, it has been shown that the relative risk for ASD in families with a previous affected child is about 18.7% [3]. Thus, there are evidences to consider that genetics might be differently involved in all ASD cases, so it is important to provide genetic counseling to all ASD families. Therefore, the genetic analysis as guarantee of success has to be considered as the first step in the protocol to study ASD.

However, the practical problem starts with the data available up today. Genetics studies of ASD have shown that in 10-15% of cases, the disorder is part of a syndrome (syndromic ASD). Of these, the most

common are fragile X syndrome (about 3%), tuberous sclerosis (2%) and several cytogenetic abnormalities as maternal duplication of 15q1-q13 (approximately 2%) or deletions 16p11 and other duplications (around 1%) [4]. More recent studies with high resolution cytogenetic techniques as aCGH have shown that about 7% of individuals with ASD present genomic copy-number variants (CNV) mainly involving genes related to synaptic cell adhesion pathways [5,6].

Moreover, recent GWAS analyses have identified candidate genes involved in neurite formation, regulation and guidance, genes related to cytoskeletal regulation and transcription (regulation of gene expression) and genes related to neurotransmission [7]. At the same time, additional candidate genes related to immune system regulations have been also described [8].

Within this multifactorial complex model, there are both gene-gene and gene-environmental factors interactions. These effects maybe the result of environmental toxic factors as well as epigenetic factors which alter several gene functions, which in turn are able to alter both the nervous system and other organs or systems that explain

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the comorbidities present in many cases beside the neurocognitive manifestations. Epigenetic effects may be affected by physical environment (eg., specific chemicals, heavy metals and toxins) or even specific types of psychological distress that alter brain chemistry, having effects of switching specific genes on or off during all the stages of child development. The ability to identify all these genetic and non-genetic risk factors and their dynamic relationship through epigenetic mechanisms is still being the challenge today. These mechanisms have the keys to establish an early successful intervention if we were able to identify adequate Epi-treating factors [9]. Today, none of the existing pharmacotherapeutic agents are effective in treating all the core symptoms of ASD, drugs like Risperidone and Aripiprazole have shown to be effective in some cases [10], whereas may induce adverse drug reactions in others [11]. Often, mainly due to negative experiences with the current treatment options and the absence of effective medical treatments which induce the chronicity of the symptoms, families turn to complementary and alternative treatment methods. The most popular complementary and alternative treatments include serotherapy (oligo elements and mega doses of vitamins and other cofactors), special restrictive diets, gastrointestinal treatments and detoxification methods as chelation, homeopathy and other alternative procedures. However, once analyzed using the scientific method, most of them have shown inconclusive results and some of them, even based on "natural" herbs, might induce potential adverse reactions in cases with impaired metabolic pathways related to their clearance [12].

Taking into account all aforesaid is easy to suppose that there might be different clinical subtypes who necessarily may have different response potentials so we have to consider that drugs and all interventions, including supplements, available nowadays might not function in the same way for all patients. Even worse, some of them may increase the risk of adverse effects in those individuals who are particularly vulnerable due to their specific pharmacogenetic profile. A large extent of this differential potential response is explained by the dynamic and interrelated combination of genetic variants present in each particular genome.

In the present paper, a new protocol that permits to choose more safety treatment options for ASD and other neurodevelopmental disorders is proposed. This protocol is based on the genomic analysis covering different pathways related to the potential response of the patient, including metabolic, immunologic, neurobiochemical and pharmacological responses.

Methodology

Bases and considerations for genomic analysis

The following sections briefly summarize the various pathways that we have to consider for a comprehensive assessment of ASD in order to determine different endophenotypes. These summaries will help to understand the content of the analysis.

These mechanisms are the following:

1. ApoE secretory pathway
2. Thrombosis
3. Methylation
4. Dopaminergic system
5. Inflammation and Immune System activity
6. Oxidative Stress enzymatic defensive system
7. Pharmacogenetics

The first three mechanisms determine the primary recovering potential because they are directly related to metabolic pathways with direct impact over the neurobiochemistry. The other three mechanisms, which act in a systemic way, secondarily act on the CNS determining the clinical outcome and prognosis in general.

ApoE secretory pathway: APOE plays an important role in the metabolism and transport of lipids. Three isoforms are encoded by the common alleles, APOE ϵ 2, APOE ϵ 3 and APOE ϵ 4 that give rise to six genotypes. ApoE2, ApoE3 and ApoE4 differ in their cysteine/arginine content at positions 112 and 158 in the receptor-binding region of ApoE. ApoE3 incorporates cysteine at codon 112 and arginine at codon 158, apoE2 contains two cysteines and shows diminished receptor binding ability, and apoE4 contains two arginine residues [13]. At the biochemical level, arginine, unlike cysteine lacks the sulphhydryl (SH) groups to potentially bind bivalent metals such as mercury, lead, copper or zinc. The different amino-acid configurations of the three apoE isoforms are potentially relevant to mercury elimination [14], where chronically exposed apoE4 carriers may accumulate metals.

In fact, increase of adverse effects in chronically exposed mercury APOE ϵ 4/ ϵ 4 and APOE ϵ 3/ ϵ 4 carriers has been reported [14,15]. Only APOE2 (with two cysteine -SH groups), and to a lesser extent APOE3 (with one -SH group), are able to bind and remove mercury from the brain and cerebrospinal fluid. Moreover, it has been also shown that amalgam sensitive individuals are significantly more likely to be carriers of the APOE ϵ 4 allele than symptom free controls [16].

APOE4 may affect neurobehavioral performance, particularly spatial memory, decades before of any clinical expression of neurodegenerative processes [17]. Finally, it has been recently shown that the strength of neuronal stress response is highly linked to the APOE genotype, pointing out the relevance of the APOE genotyping for treatment decisions [18].

Finally, an association between the genotype APOE ϵ 2/ ϵ 2 and an increased risk of cerebral palsy in infants born pre-term or at low birth weight has been also reported [19,20].

Thrombosis: Thrombophilia describes a spectrum of congenital or acquired coagulation disorders associated with venous and arterial thrombosis. These disorders can occur in the mother or in the fetus, or in both concomitantly with an incidence of 2.4 to 5.1 cases for every 100,000 births [21]. Whereas maternal thrombophilia has a higher incidence, both maternal and fetal thrombophilia can lead to thrombosis at the maternal or fetal interface. Whereas maternal thrombosis may cause severe preeclampsia, *intrauterine growth restriction*, abruption placenta, or fetal loss, fetal thrombosis can be a source of emboli that bypass hepatic and pulmonary circulation and travel to the fetal brain [21].

Perinatal stroke with an incidence of 17 to 93 cases for every 100,000 live births is defined as a cerebrovascular event that occurs between 28 weeks of gestation and 28 days of postnatal age [22-24]. Arterial ischemic stroke in the newborn accounts for 50% to 70% of cases of congenital hemiplegic cerebral palsy. Factor V Leiden and prothrombin gene mutations together with protein C, protein S, and antithrombin III deficiency have been identified in more than 50% of cerebral ischemic strokes. In addition to these thrombophilias, important risk factors for perinatal and neonatal stroke include the p.L33P mutation in the β 3 integrin gene (ITGB3) [25] and the c.20210G>A polymorphism in the coagulation factor II gene (FII) [26-28].

A large prospective study of unselected pregnant women found that c.20210G>A heterozygosity conferred a 12-fold increased risk for

placental abruption [29,30]. Moreover, in the largest reported series of c.20210G>A heterozygous children, 37% of symptomatic children had a history of arterial or venous CNS thrombosis, accounting for 30% of thromboembolic episodes and cerebral sinus thrombosis occurred in 13% of symptomatic children [31]. Finally, two meta-analyses revealed that a c.20210G>A allele is associated with a two- to threefold increased risk for intrauterine growth restriction [32,33].

Methylation pathways: Folic acid or water-soluble vitamin B9 is critical for normal cell function and for neuronal development. Folic acid is not only critical in pregnancy to prevent birth defects and to promote proper brain development [34], but it also lowers homocysteine levels. High levels of homocysteine during pregnancy may increase the risk of the child to develop brain disorders including neurodevelopment disorders [35]. The relevance of homocysteine and methylation pathways to maintain the normal brain function has also been shown for other complex neurological disorders like Alzheimer's disease [36,37].

Recently, it has been demonstrated that prenatal vitamins intake during the months before and after conception may lower the risk of having a child with ASD, at least if the mother or child carries certain genes that increase susceptibility to autism [38]. DNA analysis of mothers and children revealed that women with either one of two gene variants associated with folate regulation had increased risk of having a child with autism. This risk was completely abolished with prenatal vitamin intake around the time of conception [36]. Other recent studies have shown that mean levels of methionine, cysteine, total glutathione, and the ratio of oxidized to reduced glutathione are significantly decreased in children with ASD [39]. Finally, ASD patients present a higher rate of mutations in the methylenetetrahydrofolate reductase (MTHFR) gene and MTHFR C677T (SNPrs1801133) is a more thermolabile enzyme in vitro [40] and also a risk factor for ASD [41-43]. MTHFR with decreased activity leads to reduced methylated folic acid, essential to generate enough methyl-B12 or methylcobalamin at the rate required for normal brain function and for optimal detoxification [43].

Dopaminergic pathway: Catecholaminergic systems are involved in the regulation of aggressive behavior. Since dopamine (DA) action in the prefrontal brain area determines the effectiveness of important mental functions, such as attention and memory, this pathway is one of the most studied routes to tackle the neurochemical causes of neurodevelopmental disorders that primarily affect the prefrontal function [44].

Catechol-O-methyltransferase (COMT) plays a particularly crucial role in the regulation of prefrontal DA turnover. The common functional polymorphism Val158Met in the *COMT* gene accounts for a 4-fold variation in enzyme activity and DA catabolism [45-48]. Carriers of the valine (Val158) allele show increased COMT activity and lower prefrontal extracellular DA accompanied by weaker prefrontal neuronal activation than methionine (Met158) allele carriers. Moreover, Val158 carriers present compensatory prefrontal hyperactivation and worse performance on memory and attention tasks, but also increased limbic activation and more pain in response to unpleasant stimuli [49]. In contrast, Met158 is responsible for lower COMT activity and has been associated with elevated aggressive behavior in psychiatric patients [50,51].

The relationship between the distribution of COMT allele frequency and ADHD has been actively studied [52-55], and early-onset antisocial behavior in a high risk clinical group is predicted by the Val158/Val158 genotype [56].

Methylphenidate, the most frequently prescribed drug for the treatment of ADHD, is not effective in every case. Pharmacogenetic studies of the Val158Met polymorphism in ADHD revealed that Val158/Val158 homozygous children had significantly less severe symptoms than Met158/Met158 genotype carrying children after treatment with methylphenidate [57-59].

Enzymatic antioxidant systems: Based on abnormal metabolic profiles, it has been long hypothesized that an increased vulnerability to oxidative stress may be one of the most important factors that contribute to the development and clinical manifestation of neurodevelopment disorders [60]. In fact, an association between ASD and oxidative stress has been found [38,61-63]. Recent studies have reported low antioxidant enzyme activity in autistic children, showing that oxidative stress is part of ASD etiology [64,65]. Moreover, severity of autism depends on the relative progress in brain development prior to inundation by oxidative stress, so that early intervention is essential to guarantee optimal brain development [66].

Glutathione S-transferases (GST) are antioxidant enzymes that play an important role in cellular detoxification and the excretion of environmental pollutants including heavy metals. Glutathione S-transferase mu 1 (GSTM1) and Glutathione S-transferase theta 1 (GSTT1) are known to be highly polymorphic. Homozygous deletions of these genes result in lack of enzyme activity and impair the ability to excrete metals including mercury [67]. Combined effects of mercury accumulation and decreased levels of antioxidants have been described in some cases of ASD [68]. Especially, the association of the GSTM1 null genotype with autism has been reported by several researchers.

The relevance of oxidative stress as an important etiopathogenic mechanism consists in its synergistic interaction with other pathogenic mechanisms related to neurodegeneration, such as DNA methylation and neuroinflammation.

Immune system and inflammatory signalling pathway: Together with oxidative stress mechanisms, neuroinflammatory processes are a crucial key for most cases of ASD [69]. An aberrant neuroimmune response due to a genetic predisposition to imbalances in cytokines normal equilibrium might be the main underlying mechanism that induces an anomalous reaction in front of prenatal and postnatal environmental factors. On one hand, a disruption in the brain balance between pro- and anti-inflammatory interleukins has been described [70-72], and on the other, sustained and excessive stimulation of immunity might produce deleterious effects on nervous system function [73].

Strong support to the relationship between immune system disturbances and ASD is provided by several studies reporting that children with autism suffer from an ongoing neuroinflammatory process in different regions of the brain involving microglial activation [74-76].

Microglial over activation, as a result of an immune system deregulation, destroys synapses between intact neurons and has been shown in individuals suffering from neurological disorders caused by microglial activation [77,78]. In addition, once activated, microglia releases large amounts of nitric oxide and superoxide as a cytotoxic attack mechanism, causing additional local cellular damage by reacting with proteins, lipids, and nucleic acids.

Neuroinflammation can alter the metabolism of glutamate, an important neurotransmitter which mediates cognition and behavior. Therefore, glutamate has been directly related to ASD [79].

80]. Moreover, glutamate is a precursor of GABA, which acts as an inhibitory neurotransmitter. Low GABA levels have been found in ASD brain explaining behavioral symptoms as anxiety, irritability, hyperactivity and sleep disturbances observed in these patients. In turn, sleep disturbances interrupt the clearance of degradation products of neural activity that accumulate during wakefulness [81].

Elevated IL-6 level detected in autistic brain has been a consistent finding documented by several authors. In this sense, it has been postulated that IL-6 elevation induces disturbances in excitatory and inhibitory synaptic formations and disruption in balance of excitatory/inhibitory synaptic transmissions [82]. On the other hand, an in vitro study has recently shown that IL10 appear to induce neuronal synapse formation in cultured hippocampal neurons [83] thus, IL10 might be an important factor in synaptogenesis.

Neuroinflammation acts in synergy with the oxidative stress giving rise to a vicious circle leading to progressive loss of neuronal synapses.

Pharmacogenetic pathways: The response of ASD patients to drugs is characterized by high variability making understanding the molecular bases of pharmacological action of drugs essential.

The pharmacological action of a drug is conditioned by genetic polymorphisms that directly influence the pharmacokinetic as well as the pharmacodynamic processes. Drugs are metabolized by three main ways involving three responsible enzymes: 1) acetyltransferase enzyme NAT2 that acetylates certain sulfonamides, isoniazid, hydralazine and procainamide [84,85] and metabolizes xenobiotics; 2) a hydroxylation enzyme that catalyzes the metabolism of drugs like debrisoquine and sparteine and a large number of widely prescribed drugs; and 3) another hydroxylation enzyme that catalyzes the oxidation of phenytoin-type drugs.

Important pharmacogenetic pathways to be considered in the pharmacological treatment of ASD involve both CYP2D6 and CYP2C19. Whereas CYP2D6 supports evaluation of efficacy and safety to atomoxetine treatment [86-89], many other antidepressants are also metabolized by CYP2D6 and CYP2C19 [90-93]. Otherwise, CYP2C9 is implicated in the metabolism of antiepileptic drugs and polymorphisms in this gene explain the observed differences in its activity [93-95].

Considering neuroinflammation as a main etiopathogenic target to control in ASD patients, it is obvious that non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed in these patients [96] and taking into account that most NSAIDs might have important adverse reactions, it is highly advisable to evaluate efficacy/toxicity of NSAIDs drugs before to proceed with a prescription [97,98] and in this sense; the CYP2C9 pathway is another mechanism that we have to include in our evaluation.

Determination of genomic data

A total of 119 Spanish Caucasian patients for the preliminar analysis were recruited in the Genomic Genetics Centre. 58 children diagnosed with ASD (age, 8.6 ± 5.1 ; male-female ratio, 2:1); 23 children with Attention deficit hyperactivity disorder (ADHD) (age, 8.7 ± 5.2 ; male-female ratio, 2.5:1) and 38 children (age, 7.0 ± 3.0 ; male-female ratio, 1.5:1) with *global developmental delay*. Each affected individual had been diagnosed by board-certificated child psychiatrists according to the *Diagnostic and Statistical Manual of Mental Disorders-IV* (DSM-IV) [99]. All parents gave their informed consent to perform the genetic study.

As stated in the preceding paragraphs, ASD is a complex disorder

where many genes of different pathways may act as risk factors of varying importance for developing ASD.

To assess these pathways we analyze 44 functional SNPs of 22 genes related to 7 pathways involved in ASD pathogenesis as part of the multifactorial analysis proposed in our methodology (Table 1). Saliva/buccal cells of each patient are recollected using the OmniSwab system (Whatman™, UK). Genotyping of all SNPs is performed in the same laboratory (Progenika Biopharma, Derio, Spain) with a low-density DNA microarray based on allele-specific probes developed specifically for this purpose. Design, fabrication, validation, and analysis of the array were performed following procedures described elsewhere with minor modifications [100].

Assessment of genomic data and practical application

The present analysis evaluates specific SNPs that influence the body's response to diet and nutrition (nutrigenomics) with special attention on methylation pathways and factors influencing potential bioavailability of neurotransmitters in the prefrontal region.

In order to assess the antioxidant defense system, the state of the enzymatic mechanisms is evaluated by testing those SNPs that determine their functional capacity (Table 1).

Since increasing evidence highlights a role for the immune system in the pathogenesis of ASD, SNPs known to be involved in the overproduction of pro-inflammatory immune modulators as IL6 and IL10 have been also included in the test. Finally, individual pharmacogenetic testing is carried out in order to evaluate the potential responses, power and degree of efficacy as well as security and individual tolerance to the drugs most often prescribed in ASD.

Using this information in the context of developmental disorders, there is the opportunity to act from preconception stage, going through all stages of prenatal development, until any stage of postnatal life. The precocity in detection is the key to get success within this multifactorial framework because in predictive medicine, the earlier the detection the greater the possibility of a successful outcome to prevention and treatment.

Genomic analysis provides information to understand that there are unique cases which are conformed by a combination of several genetic factors determining a single biological profile.

Proposal of Patient Stratification according to genetic profiles

In a first visit of children with a neurodevelopmental disorder during the toddler years one of the most difficult issues to clarify is his long-term outcome. In fact, the prognosis is highly variable for all children being directly related to their sensory, motor, language, and cognitive abilities as well as the presence of comorbidities. The explanation for this inter-individual variability, which makes possible a patient stratification in different endophenotypes, has to be found at the level of each intrinsic biological potential, highly determined by the individual's underlying genetic and epigenetic modifications.

By knowing these variations it is possible to recognize various potential responses which help in setting up the bases to design more individualized intervention programs. This might be the best way to achieve better patient outcomes. If as the first step the biological response potentials can be improved, a restoration of the internal homeostasis might be closer. Then, the early intervention therapies might have a better chance for a higher success rate.

According to the genetic analysis proposed in the present method,

Pathway	Gene Name	Chromo-some	Gene symbol	SNP number	
ApoE secretory	Apolipoprotein E	19q13.2	APOE	rs429358 rs7412	
Thrombosis	Plasminogen activator inhibitor, type I (Serpine1)	7q21.3-q22	PAI-1	rs1799889	
	Integrin beta chain beta 3	17q21.32	ITB3	rs5918	
	Prothrombin (coagulation factor II)	11p11-q12	F2	rs1799963	
	Factor V Leiden	1q23	F5	rs6025	
	Coagulation factor XIII A1	6p25.3-p24.3	F13A1	rs5985	
	Methylenetetrahydro-folate reductase	1p36.3	MTHFR	rs1801133 rs1801131	
Methylation	Cystathionine-beta-synthase	21q22.3	CBS	rs234706 rs1801181 rs2298758	
	Fibrinogen, beta polypeptide	4q28	FGB	rs1800790	
	Methylenetetrahydro-folate reductase	1p36.3	MTHFR	rs1801133 rs1801131	
	Cystathionine-beta-synthase	21q22.3	CBS	rs234706 rs1801181 rs2298758	
	Catechol- O -methyltransferase	22q11.21-q11.23	COMT	rs4680	
	Vitamin D receptor	12cen-q12	VDR	rs731236 rs1544410	
Dopamine	Nitric oxide synthase 3	7q36	NOS3	rs1799983	
	Catechol- O -methyltransferase	22q11.21-q11.23	COMT	rs4680	
	Vitamin D receptor	12cen-q12	VDR	rs731236 rs1544410	
Oxidative stress	Glutathione S-transferase mu 1	1p13.3	GSTM1	rs1065411	
	Glutathione S-transferase theta 1	22q11.23	GSTT1	E5555	
	Glutathione S-transferase pi 1	11q13	GSTP1	rs1695 rs1799811	
	Superoxide dismutase 2	6q25	SOD2	rs1799725	
Inflammation y signaling	N-acetyltransferase 2	8p22	NAT2	rs15561 rs1801280 rs1799929 rs1208 rs1801279 rs1799930 rs1799931	
	Interleukin-6 (interferon β2)	7p21	IL6	rs13447446	
	Interleukin-10	1q31-q32	IL10	rs1800896	
	Pharmacogenetics	Cytochrome P450, family 2, subfamily D, polypeptide 6	22q13.1	CYP2D6	rs769258 rs1065852 rs4987138 rs5030867 rs28371724 rs28371705 rs1058164 rs1800716 rs16947
		Cytochrome P450 2C19	10q24	CYP2C19	rs4134593 rs4986893
		Cytochrome P450 2C9	10q24	CYP2C9	rs1799853 rs1057910
	N-acetyltransferase 2	8p22	NAT2	rs15561 rs1801280 rs1799929 rs1208 rs1801279 rs1799930 rs1799931	

Table 1: Polymorphisms of different genes involved as determinants in the pathways which functionality is to be analyzed.

considering the integrated effect of several genotypes at some specific pathways, it is possible to estimate the neuroplasticity potential and at the same time, to determine possible prognosis modifier factors.

Profiles of neuroplasticity potential: Three possible potentials of neuroplasticity, low, medium-grade or high-grade, might be established considering the effect of several gene combinations at three different pathways (Table 2). Whereas the ApoE secretory pathway includes the different APOE gene variants, the methylation pathway is represented by MTHFR and CBS gene polymorphisms and the status of COMT V158M affects the neurotransmitter balance. Since these pathways are closely linked, they cannot be analyzed separately but have to be examined as a whole [42]. Their dynamic relationship (epistatic interaction) must be always considered before making a decision.

To define the different potentials of neuroplasticity, four considerations should be taken into account. First, neurons in the CNS are plentiful in ApoE receptors that function facilitating the transport of synaptogenic cholesterol from astrocytes to neurons [101]. At the same time they are signaling receptors for a ligand known as reelin. Reelin is a neuronal regulator for migration during embryonic development, as well as for neurotransmission and has been also postulated as a candidate gene for ASD [102]. ApoE4 reduces the ApoE receptor expression and thus, it acts as an interfering agent affecting reelin function independently of the genotype at the reelin locus [103]. Therefore, there are reasons to suppose that ApoE4 might interfere at two different stages: during embryonic neurodevelopment and during postnatal synaptogenesis.

Second, MTHFR is a crucial key player to ensure the proper functioning of the folic acid metabolic cycle which in turn is a critical

mechanism to support relevant cellular functions such as nucleotide synthesis and methylation. In folate deficiency, the flux through the methylation cycle is decreased [40] and disturbances in methylation status can alter the expression pattern of some genes directly related to ASD [104].

Third, the transsulfuration pathway from homocysteine to cystathionine which is catalyzed by cystathionine beta synthase (CBS) is tightly related to the methylation pathway. Disturbances in the transsulfuration pathway raise homocysteine levels with deficiencies in methionine leading to a reduced methylation capacity causing directly the alteration of epigenetic mechanisms.

Fourth, COMT is another important piece of this complex process and its functioning has to be evaluated not only at the level of protein structure (valine or methionine) but also at the expression level regulated by epigenetic methylation and genetic control of methylation via MTHFR [105].

The combination of the different genotypes of these genes exerts a direct effect on neuroplasticity. The different combinations define three different grades of neuroplasticity potential and are shown in Table 2.

Genetics as prognosis modifiers: Combinations of genes at three pathways with prognostic importance for ASD patients have been included in the test to define the prognosis for each patient. These combinations, acting in synergy with those discussed above, may have an impact on the clinical phenotype and outcome severity, including particular vulnerability to the most frequent comorbidities.

There are three main pathways which might determine differential

Neuroplasticity potential		pathway	gene	alleles/genotype
LOW	A	ApoE secretory	ApoE	genotypes 4/4; 2/2
		Methylation	MTHFR	Val/Val
			CBS	833 CC, 844ins68 II
MEDIUM-GRADE	A	Dopaminergic	COMT	Met/Met
		ApoE secretory	APOE	genotypes 2/4; 3/4
			MTHFR	Val/Val
	B	Methylation	CBS	833 TT, 844ins68 II
			Dopaminergic	COMT
		ApoE secretory	APOE	genotypes 2/4; 3/4
HIGH-GRADE	A	Methylation	MTHFR	Ala/Ala
			CBS	833 CC, 844ins68 II
		Dopaminergic	COMT	Val/Met
	B	ApoE secretory	APOE	Genotypes 2/3; 3/3
			MTHFR	Val/Val
		Methylation	CBS	833 TT, 844ins68 II
	Dopaminergic		COMT	Val/Val
	C	ApoE secretory	APOE	Genotypes 2/3; 3/3
			MTHFR	Ala/Ala
Methylation		CBS	833 CC, 844ins68 II	
	Dopaminergic	COMT	Val/Val	

Table 2: Profiles of neuroplasticity potential.

susceptibility to comorbid health problems. The variable degree of impairment at these pathways might be used as prognostic factors which would determine the clinical outcome.

These pathways are the following:

1. Genetic factors involved in the immune and inflammatory responses that have been also linked to ASD pathogenesis.
2. Genetic factors which determine the effectiveness of the main cellular defence mechanisms against oxidative stress.
3. Pharmacogenetics that might determine the dose and security for drug prescription.

The combination of results obtained at these pathways can give three possible outcomes:

1. **Poor prognosis** (Table 3), for patients combining the higher pro-inflammatory tendency with the lower antioxidant tolerance being slow-metabolizers at the pharmacogenetic pathways CYP2D6 and CYP2C9.
2. **Intermediate prognosis**, for patients combining a medium-grade pro-inflammatory tendency with a medium-grade toxic tolerance and being intermediate metabolizers at the pharmacogenetic pathways CYP2D6 and CYP2C9. Intermediate prognosis could also be divided into low-grade-intermediate and high-grade-intermediate.

a. Low grade intermediate prognosis (Table 4a) would be represented by intermediate functional limitation of the inflammatory pathway just involving one of the interleukins, total or intermediate functional limitation involving 2 detoxification pathways distinct from GSTM1 or GSTT1 and fast or intermediate pharmacogenetic profile at CYP2C9 and CYP2D6.

b. High grade intermediate prognosis (Table 4b) would show total functional limitation of one or intermediate functional limitation of both interleukins, total functional limitation involving 2 detoxification pathways and intermediate pharmacogenetic profile at CYP2C9 and CYP2D6.

3. **Good prognosis** (Table 5), for patients combining the lower pro-inflammatory tendency with the higher antioxidant tolerance being fast-metabolizers at the pharmacogenetic pathways CYP2D6 and CYP2C9.

By combining the three profiles of neuroplasticity potentials with the three prognosis modifier factors, nine main different clinical subtypes can be outlined (Figure 1).

Examples of treatment adjustments according to the genomic profile: Today, treatment options for ASD as well as for the majority of NDD are controversial and contradictory. The most plausible reason for this confusion lies in the great causal heterogeneity inherent in these disorders. Conflicting results will be solved when more rigorous studies

Pathway	Gene	Locus	Allele/genotype				Comment
			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	
Inflammatory signalling	IL6		GG	GG	CG		High inflammatory tendency
	IL10		AA	AG	AA		
Oxidative stress			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Low toxic tolerance
	GSTM1		Null	Null	Null	Present	
	GSTT1		Null	Null	Present	Null	
	GSTP1	105	Val/Val	Ile/Val	Val/Val	Val/Val	
		114	Val/Val	Ala/Val	Val/Val	Val/Val	
SOD2		Ala/Val; Val/Val	Ala/Val; Val/Val	Ala/Val; Val/Val	Ala/Val; Val/Val		
NAT2		*5A/*5A; *6A/*6A; *7A/*7A; *5A/*6A; *5A/*7A; *6A/*7A	*4/*6A; *4/*5A; *4/*7A; *5A/*5A; *6A/*6A; *7A/*7A; *5A/*6A; *5A/*7A; *6A/*7A	*5A/*5A; *6A/*6A; *7A/*7A; *5A/*6A; *5A/*7A; *6A/*7A	*5A/*5A; *6A/*6A; *7A/*7A; *5A/*6A; *5A/*7A; *6A/*7A		
Pharmacogenetics	CYP2D6		*3/*3; *4/*4; *5/*5; *6/*6; *3/*4; *3/*5; *3/*6; *4/*5; *4/*6				Poor metabolizer
	CYP2C9		*2/*2; *3/*3; *2/*3				

Table 3: Genetic profile of patients with poor prognosis.

Pathway	Gene	Locus	Allele/genotype					Comment
			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	
Inflammatory signalling	IL6		CG	CC				Medium-low grade inflammatory tendency
	IL10		GG	AG				
Oxidative stress			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Medium-grade toxic tolerance
	GSTM1		Present	Present	Present	Present	Present	
	GSTT1		Present	Present	Present	Present	Present	
	GSTP1	105	Ile/Val	Ile/Val	Val/Val	Val/Val	Val/Val	
		114	Ala/Val	Val/Val	Ala/Val	Val/Val	Val/Val	
SOD2		Ala/Val	Ala/Ala	Ala/Ala	Ala/Ala;	Val/Val		
NAT2		*4/*6A; *4/*5A; *4/*7A	*4/*6A; *4/*5A; *4/*7A; *5A/*7A; *6A/*7A	*4/*6A; *4/*5A; *4/*7A; *5A/*7A; *6A/*7A	*4/*6A; *4/*5A; *4/*7A; *5A/*7A; *6A/*7A	*4/*6A; *4/*5A; *4/*7A		
Pharmacogenetics	CYP2D6		*1/*1; *2/*2; *3/*3; *4/*4; *5/*5; *6/*6; *3/*4; *3/*5; *3/*6; *4/*5; *4/*6					Fast or Intermediate metabolizer
	CYP2C9		*1/*1; *2/*2; *3/*3; *2/*3					

Table 4a: Genetic profile of patients with low grade medium-term prognosis.

Pathway	Gene	Locus	Allele/genotype					Comment
			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	
Inflammatory signalling	IL6		GG	CC	CG			Medium- pro-inflammatory tendency
	IL10		GG	AA	GA			
Oxidative stress	GSTM1		Null	Null	Present	Present	Present	Medium-grade toxic tolerance
	GSTT1		Present	Present	Null	Null	Present	
	GSTP1	105	Val/Val	Ile/Val	Val/Val	Ile/Val	Val/Val	
		114	Ala/Val	Val/Val	Ala/Val	Val/Val	Val/Val	
	SOD2		Ala/Val	Ala/Val	Ala/Val	Ala/Val	Val/Val	
NAT2		*4/*6A; *4/*5A; *4/*7A	*4/*6A; *4/*5A; *4/*7A	*4/*6A; *4/*5A; *4/*7A	*4/*6A; *4/*5A; *4/*7A	*5A/*7A; *6A/*7A		
Pharmacogenetics	CYP2D6		*1/*3; *1/*4; *1/*5; *1/*6; *2/*3; *2/*4; *2/*5; *2/*6					Intermediate metabolizer
	CYP2C9		*1/*2; *1/*3					

Table 4b: Genetic profile of patients with high-grade medium-term prognosis.

Pathway	Gene	Locus	Allele/genotype				Comment
			Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	
Inflammatory signalling	IL6		GG	GG	CG		Low-grade pro-inflammatory tendency
	IL10		GG	GA	GG		
Oxidative stress	GSTM1		Present	Present	Present	Present	High-grade toxic tolerance
	GSTT1		Present	Present	Present	Present	
	GSTP1	105	Ile/Ile	Ile/Ile	Ile/Ile	Ile/Val	
		114	Ala/Ala	Ala/Val	Ala/Val	Ala/Ala	
	SOD2		Ala/Ala	Ala/Ala	Ala/Val	Ala/Val	
NAT2		*4/*4	*4/*4; *4/*6A; *4/*5A; *4/*7A;	*4/*4; *4/*6A; *4/*5A; *4/*7A;	*4/*4; *4/*6A; *4/*5A; *4/*7A;		
Pharmacogenetics	CYP2D6		*1/*1; *2/*2				Rapid metabolizer
	CYP2C9		*1/*1				

Table 5: Genetic profile of patients with good prognosis.

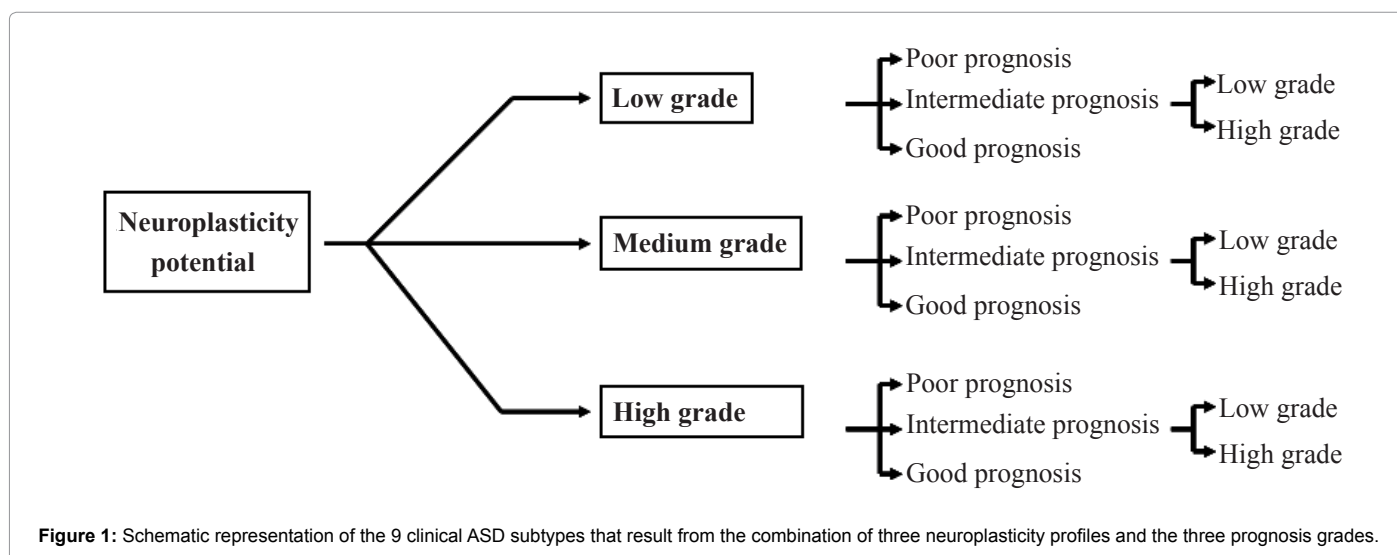


Figure 1: Schematic representation of the 9 clinical ASD subtypes that result from the combination of three neuroplasticity profiles and the three prognosis grades.

will be performed where well characterized subgroups of patients substitute one single group.

For that reason, a preliminary outline of the clinical subtypes is proposed in the present paper as the first step to decide on more rational and effective treatments for ASD patients.

Example of supporting recommendations for the profile of lower neuroplasticity potential combined with the poorer prognosis: Patients with this profile show the greatest tendency to immune deregulation, inflammation, oxidative stress and metabolic dysfunctions which induce mitochondrial dysfunction. The most vulnerable patients presenting the worst outcome belong to this clinical subtype. Most

of them have seizures and other neurological symptomatology, and they also represent the group of patients at higher risk to develop several comorbidities as neuroinflammation, immune disorders and a distinctive inflammatory bowel disease [106], among others.

It has been demonstrated that three month treatment with methylcobalamin and folic acid may induce a significant improvement in behavioral symptoms associated with ASD in a group of children with abnormal redox and methylation metabolism [107]. Due to their methylation and sulphur disturbances as well as higher oxidative stress level these cases are extremely vulnerable to toxic agents, even at very low doses which commonly are not hazardous for the rest of the population. Therefore, this profile might be considered as the risk profile for post vaccine reactions [108].

The treatment to be avoided or to be prescribed under careful monitoring for toxicity includes amphetamines, methylphenidate, risperidone as well as CYP2D6 inhibitors as haloperidol and valproic acid, all them frequently used as part of the ASD pharmacological treatment [109,110]. Supplements that provide methyl groups, such as DMG or TMG, are commonly used by practitioners of biological medicine, but must be administered with caution [111].

Nevertheless, it is important to take into account that for this ASD subtype vitamin/mineral supplementation may be highly recommended to improve an impaired nutritional and metabolic status [112].

Another important pillar for the treatment of these cases is based on the anti-inflammatory treatment. At this point, it is important to keep in mind that the most commonly used anti-inflammatory medication (e.g. steroids), or immunomodulators (e.g. immunoglobulins) do not have any effect on neuroglial activation because these drugs are not able to act on the central nervous system's (CNS) innate immune system disturbances. These drugs were designed to work on adaptive immunity and effective therapies to control neuroinflammation might involve modifying neuroglial responses in the brain [113].

At the present, therapeutical options include NSAIDs and selective COX-2 inhibitors with demonstrated efficacy in controlling brain inflammation [114] just for the ASD subtypes with intermediate or good prognosis.

For ASD, especially for the low neuroplasticity and poor prognosis subtype, the challenge is to use drugs with pleiotropic actions. In this sense, a new class of anti-inflammatory drugs is emerging. An example is a Vitamin D binding protein macrophage activating factor known as GcMAF which might be indicated just in children with immune imbalances [115]. Another well-known drug, suramin, has been medically used for the treatment of African sleeping sickness since early 20th century and is an ATP mediator. It is well known that ATP is part of a signaling system linked to mitochondria and critical for innate immunity. Recently, it has been reported that this ATP mediator is able to normalize brain synapse structure, cell-to-cell signaling, social behavior and motor coordination as well as to recover mitochondrial metabolism in an animal model of Autism [116]. These are examples of drugs which are opening promising doors to new therapeutic strategies for specific ASD subtypes.

Considering that disturbances in redox metabolisms may be a main factor to control in the low neuroplasticity and poor prognosis ASD subtype, anti-oxidant protection is crucial for the treatment in this group. Several recent studies have reported that N-acetyl cysteine (NAC) is the best way to improve the glutathione levels and, it may

restore glutamate homeostasis reducing the excitability of the glutamate system by stimulating inhibitory receptors [117-119]. So, NAC is a good candidate as a first line treatment for these patients.

Conclusions

ASD is a highly multifactorial disorder. In this multifactorial context, many other for the moment unknown genetic factors as well as many non-determined environmental factors modifying the expression of these genes over the time could exist. It is important to bear in mind that analyzing a complex phenomenon of polygenic and multifactorial nature, the results of the present test do not have an absolute value.

Instead, the genetic test proposed in the present method gives the possibility to classify the patients by their susceptibility to different genetically determined and environmental factors. The application of this method permits the establishment of two main, closely linked profiles, the profile of neuroplasticity potential and the profile of prognosis modifiers that taken together bear at least nine different clinical subtypes. Knowing the genetic basis of each clinical subtype, an adequate treatment can be designed for each patient taking into account his own biological profile.

Taken together, the use of the proposed method allows the early identification of the heterogeneity represented by different clinical subtypes and, in consequence the design of more specific treatment strategies. Once recognized this singularity which is determined by the particular genetic makeup of each case, it is possible to modulate the effect of environmental factors, as well as the evolution and prognosis of the disease.

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