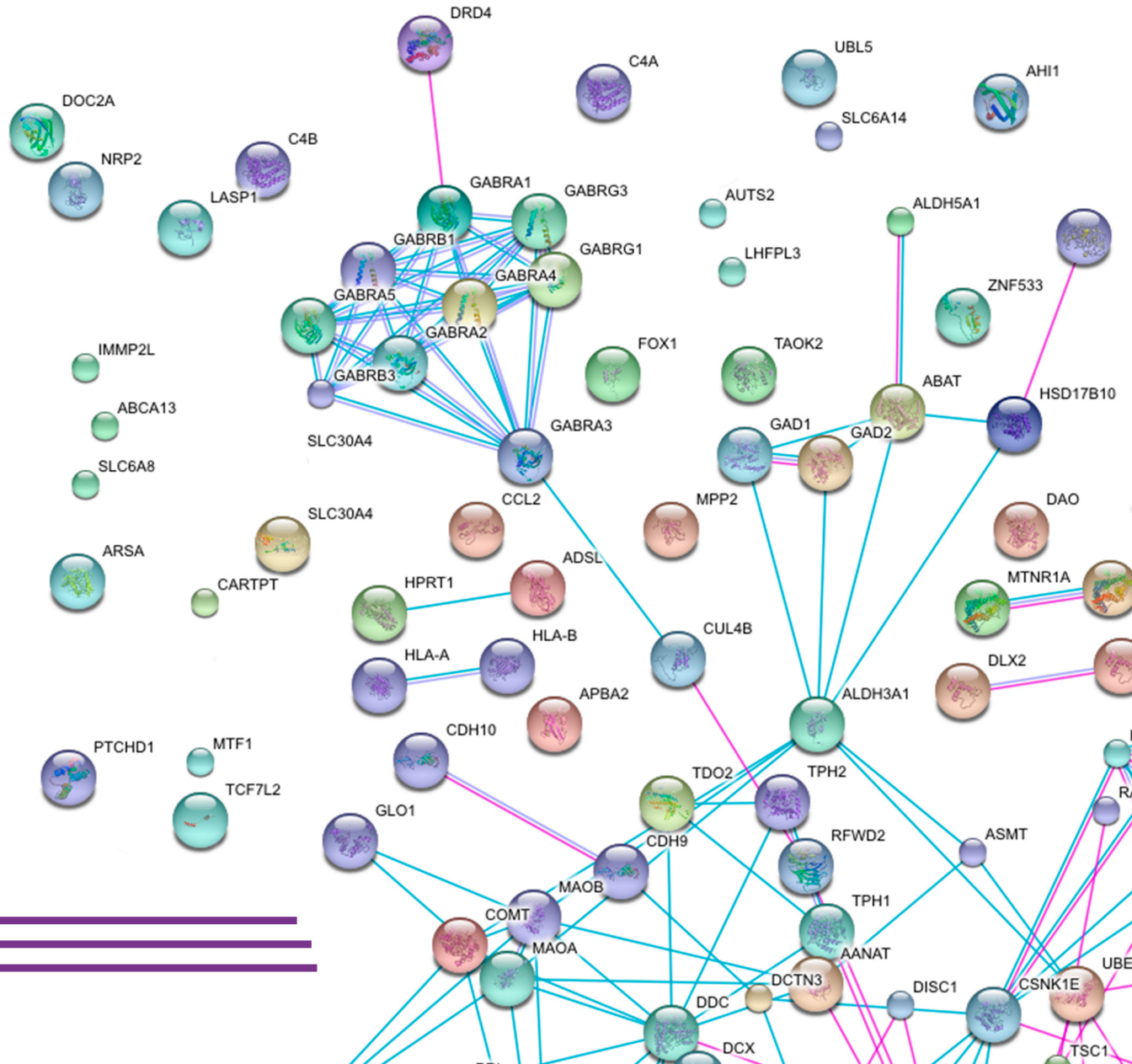
Universidad de Jaén

AUTISM SPECTRUM DISORDER: MOLECULAR PROFILING ANALYSIS AND IDENTIFICATION OF CANDIDATE GENES THROUGH COMPLEX SYSTEMS BIOLOGY APPROACHES

LETICIA DÍAZ BELTRÁN



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Departamento de Biología Experimental

Autism spectrum disorder: molecular profiling analysis and identification of candidate genes through complex Systems Biology approaches

Ph.D. Thesis

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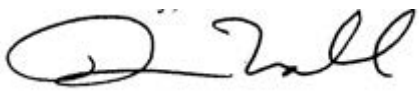

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Abstract

Science is always worth it because its discoveries, sooner or later, are always applied.

Severo Ochoa de Albornoz

Autism spectrum disorder (ASD) refers to a broad range of neurological and developmental conditions characterized by challenges with social skills, repetitive behaviors, speech and nonverbal communication. Evidence has shown that there is not one autism but many subtypes, most influenced by a combination of genetic, neurological, immunological and environmental factors. Several reasons may cause the development of autism, and it is often accompanied by a substantial burden of comorbidity. The tremendous clinical and etiological variability between individuals with ASD has made systems biology and precision medicine the most promising approaches in the search of efficient treatments.

In this Doctoral Thesis, different strategies of the emerging field of systems biology are explored to better understand the etiopathogenesis of ASD, with the aim of improving how autism is currently diagnosed and contributing to decipher its clinical and neurobiological heterogeneity by using genome-wide search for autism candidate genes. Our main goal is to disentangle the complexity of ASDs underlying neurological mechanisms, overlapping of genes and symptoms, comorbidities with other neurological conditions and possible differential evolutionary constraints, in order to identify novel genes and biological pathways that may specifically impact functional outcomes, contributing to advance in the field of personalized medicine.

First, by conducting an integrated systems biology analysis of 9 independent gene expression experiments, we found a set of 66 genes that appeared to be non-random, differentially expressed in blood and brain and of potential etiologic relevance to autism as they had enriched roles in neurological processes. This result suggests that there is a detectable autism

signature in the blood that may be a molecular echo of ASD-related regulatory impairment in the brain and might help as a biomarker for early diagnosis.

Secondly, we performed a cross-disorder analysis of autism and its related conditions to identify genes unique to ASD that may play a more causal role in this disorder and a genomic evolutionary rate profiling analysis to explore their evolutionary history. Our findings revealed a core set of 262 genes specific to autism, with roles in basal cellular functions that have been subjected to significantly less evolutionary constraints than the autism genes shared with other comorbid conditions.

Finally, we devised a two-fold comparative analysis of autism with sibling comorbid conditions to define a multi-disorder subcomponent of ASD and predict novel candidates validated through high throughput transcriptome expression profiling studies. By leveraging prior knowledge of disease-related biological processes and interaction networks of autism sibling conditions, a defined set of 19 genes not previously linked to ASD that were significantly differentially regulated in individuals with autism was found. Furthermore, this gene set had potential etiologic importance to autism, given its crucial role in neurological processes critical for optimal brain development and function, learning and memory, cognition and social behavior.

Overall, our systems biology approach represents a novel perspective of autism from its integrated analysis of independent gene expression studies involving various tissue types and from the point of view of related comorbid conditions. This devised strategy has advanced our understanding of the molecular systems involved in the pathology of ASD and may enlighten and focus the genome-wide search for autism candidate genes to better define the genetic heterogeneity of this spectrum disorder and contribute in the ongoing work to build autism biomarkers to help move towards precision medicine.

Resumen

La ciencia siempre vale la pena porque sus descubrimientos, tarde o temprano, siempre se aplican.

Severo Ochoa de Albornoz

Los trastornos del espectro autista (TEA) hacen referencia a una amplia gama de afecciones neurológicas y del desarrollo caracterizadas por alteraciones en las habilidades sociales, conductas repetitivas, habla y comunicación no verbal. Se ha demostrado que no hay un solo grado de autismo sino muchos subtipos, la mayoría influenciados por una combinación de factores genéticos, neurológicos, inmunológicos y ambientales. Varias causas pueden influir en el desarrollo del autismo y, a menudo, va acompañado de una carga sustancial de comorbilidad. La enorme variabilidad clínica y etiológica entre los individuos con TEA ha hecho que la biología de sistemas y la medicina de precisión sean los enfoques más prometedores en la búsqueda de posibles tratamientos más eficaces.

En esta Tesis Doctoral se exploran diferentes estrategias propias de la biología de sistemas para comprender mejor la etiopatogenia de los trastornos del espectro autista, con el objetivo de mejorar la forma en que se diagnostica actualmente y contribuir a descifrar su heterogeneidad clínica y neurobiológica, mediante la búsqueda de genes candidatos a autismo en todo el genoma humano. Nuestro principal objetivo es desentrañar la complejidad de los mecanismos neurológicos subyacentes a los TEA, el solapamiento de genes y síntomas, las comorbilidades con otras afecciones neurológicas y las posibles limitaciones evolutivas diferenciadoras, a fin de identificar nuevos genes y rutas biológicas que puedan repercutir específicamente en los resultados funcionales, contribuyendo así a avanzar en el campo de la medicina personalizada.

En primer lugar, al realizar un análisis integrado de 9 experimentos de expresión génica independientes entre sí, encontramos un conjunto de 66 genes que parecían no ser aleatorios y que serían de potencial relevancia etiológica para el autismo, ya que tenían un papel muy

destacado en procesos neurológicos importantes y se expresan de forma simultánea en sangre y cerebro. Este resultado sugiere la existencia de una firma biológica propia del autismo, detectable en los componentes sanguíneos de personas con trastorno del espectro autista, que puede servir como señal de una disfunción neurológica cerebral y ayudar como biomarcador en el diagnóstico precoz.

En segundo lugar, se realizó un análisis transversal del autismo y otros trastornos relacionados para identificar los genes exclusivos de los TEA que pueden desempeñar un papel más causal, así como un análisis de secuencias para explorar su historia evolutiva. Nuestros hallazgos revelaron un conjunto significativo de 262 genes específicos de autismo, implicados en funciones celulares basales y que han sido sometidos a limitaciones evolutivas significativamente menores que los genes compartidos entre los trastornos del espectro autista y los demás trastornos concurrentes.

Por último, se llevó a cabo un análisis comparativo del autismo con sus condiciones comórbidas más estrechamente relacionadas para definir un subconjunto de genes compartidos entre éstas comorbilidades y los TEA, y así predecir nuevos genes candidatos validados mediante estudios de perfiles de expresión génica. Aprovechando el conocimiento previo de los procesos biológicos relacionados con estos trastornos y las redes de interacción de las enfermedades comórbidas más ligadas a autismo, se encontró un conjunto definido de 19 genes no vinculados previamente a los TEA que se expresaban de forma diferencial en individuos con autismo. Además, estos genes podrían ser de una posible importancia etiológica para el autismo, dado su papel crucial en procesos neurológicos críticos para el correcto desarrollo y funcionamiento del cerebro, el aprendizaje y la memoria, la cognición y el comportamiento social.

En general, este enfoque de la biología de sistemas representa una perspectiva novedosa del autismo desde su análisis integrado en estudios independientes de expresión génica en varios tipos de tejido y desde el punto de vista de sus comorbilidades. Esta estrategia diseñada resulta útil para comprender mejor los mecanismos moleculares que intervienen en

los TEA, y se centra en la búsqueda de genes candidatos a autismo que nos permitan definir mejor su compleja arquitectura genética y así contribuir a la identificación de biomarcadores de autismo que ayuden a avanzar hacia la medicina de precisión.

Contents

Chapter 1

Ph.D. Thesis Report

Las neuronas son como misteriosas mariposas del alma, cuyo batir de alas quién sabe si esclarecerá algún día el secreto de la vida mental.

Neurons are like mysterious butterflies of the soul, whose flapping wings who knows whether they will one day unlock the secret of mental life.

Santiago Ramón y Cajal

This Doctoral Thesis is presented as a collection of articles, that is, in the format of a compilation thesis, in accordance with the provisions of point 3.d of article 23 of the *Regulations for Doctoral Studies from the University of Jaén*. This chapter summarizes and integrates the content of the publications included in Chapter 2 of this Doctoral Thesis Report.

In this chapter we begin by introducing the theme of the Doctoral Thesis and the existing problems (Section 1.1), then we explain the background, state of the art and the bioanalytical approaches on which the work is based (Section 1.2). Afterwards, the research aim that leads to the development of this Doctoral Thesis is presented (Section 1.3), and the objectives established are described (Section 1.4). Subsequently, the results obtained are discussed (Section 1.5) and, finally, the general conclusions drawn from the Doctoral Thesis

are presented, as well as the lines of future work (Section 1.6). The list of abbreviations can be found in section 1.7.

1.1 Introduction

The Doctoral Thesis presented in this report has its origin in a research project whose main objective is to take advantage of different systems biology approaches to prioritize genome-wide search for autism spectrum disorder (ASD) candidate genes. The main goals are to find a common molecular signature that may be useful as a diagnostic biomarker and to propose a model that leverages prior knowledge from the point of view of related biological processes and interaction networks of autism comorbid conditions to predict genotype-phenotype signatures and reveal different evolutionary constraints.

Autism spectrum disorder is a complex, pervasive, neurobiological condition that involves an intricate interplay of both genetic and environmental risk factors, with immune alterations and synaptic connection deficiency in early life [1]. Although its pathophysiology remains unknown, ASD appears to be a highly heritable disorder, where de novo mutations, common variants and short nucleotide polymorphisms identified in autistic individuals account for approximately 50% of cases [2, 3].

This condition covers a wide spectrum of mental disorders of the neurodevelopmental type, ranging from individuals with severe impairments (who may be silent, developmentally disabled, and prone to frequent repetitive behavior) to high functioning individuals (who may have active but distinctly odd social approaches, narrowly focused interests, and verbose, pedantic communication) [4]. Concurrent comorbidities may appear at any time during child development, although some might not occur until later in adolescence or adulthood.

Treatment options are still limited to ameliorate ASD symptomatology, including both symptoms related to diagnostic criteria and those considered to be a function of comorbid and medical conditions known to exacerbate the severity of the presentation [5]. In some cases,

comorbid disorders affect the efficacy of therapeutic interventions, therefore, it is relevant for a treatment outcome to identify the concurrent conditions and treat them separately. The identification of effective treatments for ASD must face numerous challenges since genetic, environmental, cognitive and social heterogeneity in the autistic phenotype may produce high variability in study samples that reduce the potential effect size of an intervention [6, 7, 8, 9]. Other factors, such as small sample sizes, lack of significantly impaired study participants and the use of outcome measures not standardized, also contribute to the difficulties of identifying useful therapies [9].

Autism is the most rapidly increasing developmental disability with enormous costs to individuals and to society. The importance of modeling autism research cannot be overstated. Identification of subgroups would aid in both research and treatments. This subtyping can be done on the basis of genes or clinical data. Subgrouping the population might result in subtypes that have distinctive symptoms and pathology that are already familiar in the medical literature, and can draw upon treatments that work in existing treatable conditions. Biomarkers can be used for clustering subgroups. Many of the genetic, metabolic, immunologic, proteomic and anatomical differences found in autistic individuals can be used for subtyping this disorder [10].

The hallmark heterogeneity of ASD should not hamper the understanding of autism subgroups and associated biomarkers of pathological states that are necessary to make progress in this research field. The complexity of autism poses both a challenge and an opportunity for a systems biology perspective, since the identification of objective rather than subjective diagnostic measures and the possibility of biological signatures contributing to the classification of ASD will impulse the quest for precision medicine and personalized therapeutic treatments in this diverse population [5].

1.2 Background

1.2.1 Definition

In 1943, child psychiatrist Leo Kanner reported 11 children, eight boys and three girls, who were highly intelligent but displayed "a powerful desire for aloneness" and "an obsessive insistence on persistent sameness", among these children was included 5-year-old Donald who was "happiest when left alone, almost never cried to go with his mother, did not seem to notice his father's home-comings, and was indifferent to visiting relatives... wandered about smiling, making stereotyped movements with his fingers... spun with great pleasure anything he could seize upon to spin....Words to him had a specifically literal, inflexible meaning... When taken into a room, he completely disregarded the people and instantly went for objects" [11]. Almost simultaneously, in 1944, pediatrician Hans Asperger identified a behavioral pattern in four boys that involved "a lack of empathy, little ability to form friendships, one-sided conversations, intense absorption in a special interest, and clumsy movements", included among them was 6-year-old Fritz, who "learnt to talk very early... quickly learnt to express himself in sentences and soon talked "like an adult" ... never able to become integrated into a group of playing children... did not know the meaning of respect and was utterly indifferent to the authority of adults... lacked distance and talked without shyness even to strangers... it was impossible to teach him the polite form of address... Another strange phenomenon... was the occurrence of certain stereotypic movements and habits" [12].

These pioneer primal reports [11, 12] brightly portray what we now define as autism or the autism spectrum disorder. The spectrum has a wide range, encompassing original Kanner's syndrome (firstly called autistic disturbances of affective contact) and Asperger's syndrome (originally entitled autistic psychopathy in childhood). Perception of autism has substantially evolved in the past 73 years, with an exponential increase in research from mid-1990s [13]. Over the past decades, rapid advances in our understanding of autism symptoms and the multifactorial origin of ASD, where genetics plays a key role in combina-

tion with developmentally early environmental factors, are challenging traditional nosology and have stimulated the efforts to standardize the measurement of autism traits in order to re-conceptualize and stablish appropriate clinical thresholds for early diagnosis [14].

Since the former view of autism as a type of childhood psychosis is no longer sustained and prior standard definitions became obsolete as new research outcomes arise, the more pressing need for early identification, targeted therapeutic intervention and personalized medicine approaches to specific autistic syndromes led to the major and latest revision of the fifth edition of the Diagnostic Manual of Mental Disorders (DSM-5) [15], published in May 2013, that represents an evolution in the diagnosis of ASD, as it encompasses several recent noteworthy scientific discoveries made in autism etiopathology, providing an evolved and more accurate definition of the diagnostic criteria for autism spectrum disorder [13].

Therefore, autism is nowadays considered as a set of lifelong and disabling heterogeneous neurodevelopmental conditions that adopted the umbrella term autism spectrum disorder, with no definition of subtypes, since the previous (DSM-IV-TR) diagnosis of autism [16], Asperger syndrome, pervasive developmental disorder not otherwise specified (PDD-NOS) and childhood disintegrative disorder are embraced in the updated DSM-5 redefinition. The core features of ASD include early onset persistent impairments in reciprocal social interaction, communication skills and unusually restricted, repetitive and stereotyped patterns of behaviors, interests or activities, sensory issues, and sometimes, cognitive delays (Figure 1.1).

ASD strikes each subject in a different manner and at distinct levels of severity, thus, some autistic individuals severely affected by the condition are not able to speak, require continuous one-on-one care and are never capable of having a self-sufficient lifestyle, whereas others who present less severe manifestations of the disorder are able to communicate, and, ultimately, develop the necessary skills to lead independent and fulfilling lives [17]. Generally, individuals affected with autism present abnormal cognitive profiles, such as difficulties in social cognition and perception, executive function deficit and anomalous comprehension

ASD Features	
Core features in DSM-5 criteria*	
Persistent deficits in social communication and social interactions across multiple contexts	Deficits in social-emotional reciprocity Deficits in non-verbal communicative behaviours used for social interaction Deficits in developing, maintaining and understanding relationships
Restricted, repetitive patterns of behavior, interests or activities	Stereotyped or repetitive motor movements, use of objects or speech Insistence on sameness, inflexible adherence to routines or ritualized patterns of verbal or non-verbal behavior Highly restricted, fixated interests that are abnormal in intensity or focus Hyper-reactivity or hypo-reactivity to sensory input or unusual interest in sensory aspects of the environment
Associated features not in DSM-5 criteria	
Atypical language development and abilities	Age<6 years: frequently deviant and delayed in comprehension; two-thirds have difficulty with expressive phonology and grammar Age≥6 years: deviant pragmatics, semantics and morphology, with relatively intact articulation and syntax (ie, early difficulties are resolved)
Motor abnormalities	Motor delay; hypotonia; catatonia; deficits in coordination, movement preparation and planning, praxis, gait and balance
Excellent Attention to detail	

Figure 1.1: Behavioral characteristics of ASD [11, 12]

and processing of information. These profiles are supported by deviant neurodevelopment at the systems level [13].

1.2.2 Epidemiology

1.2.2.1 *Prevalence*

Autism spectrum disorder prevalence rate has been consistently rising since the first epidemiological studies [18, 19], which reported that 4.5 per 10000 individuals in the UK suffered from autism, being boys more common than girls in a ratio of 2.6 to 1. The increase is probably partly due to changes in diagnostic concepts and criteria [20].

Regarding the prevalence of ASD, data arisen from developed countries appear to be more comprehensive and reliable, in comparison with those from developing nations. De-

spite this, one fact that can no longer be denied is that, as it stands today, ASD occurs globally irrespective of cultural and geographical boundaries or degree of industrialization; with variable grades of tangible data from diverse regions worldwide where studies had been carried out [21].

Nevertheless, the number of autism diagnoses has continued to rise in the past two decades and appears to be increasing globally. To most experts in autism and autism epidemiology, the biggest factors accounting for the boost in autism prevalence are the shifting definitions and improved awareness about the disorder (recognition, younger age of diagnosis and better diagnostic tools), although an increase in risk factors cannot be ruled out [22, 23]. Early studies also reported that autism affects more males than females in a 4.5/1 ratio [22, 23]. Autistic females may have been under-recognized [24]. Empirical data point out a diagnostic bias towards males, suggesting that high-functioning females have a later diagnosis in comparison to males [13, 25, 26, 27]. This could mean that females would need more concomitant behavioral or cognitive conditions than males do to be clinically diagnosed. This bias in diagnosis may occur as a result of behavioral criteria for ASD or gender stereotypes, and might reflect better compensation of the so-called camouflage in females [13, 28, 29, 30, 31, 32]. Nonetheless, male predominance represents a consistent epidemiological outcome with etiological implications. It may suggest female-specific protective effects, so that females would need to have a greater genetic or environmental burden than would males to reach the diagnostic threshold. These protective effects would imply that relatives of female probands would have a higher risk of ASD or at least more autistic features than would have relatives of male probands [13, 33]. Alternatively, male-specific risks could increase susceptibility [13, 24, 34]. Thus, the existence of gender-linked etiological burden and susceptibility highlights the relevance of stratification by sex, and of male-female comparisons to unravel the etiological role of gender-linked factors at genetic, endocrine, epigenetic and environmental levels [13].

Nowadays, it is estimated that worldwide 1 in 160 children has an ASD. This appraisal represents an average figure, and stated prevalence differs considerably across studies [35, 36].

Some large-scale, well-controlled surveys have indeed reported figures that are substantially higher, especially in areas where researchers have full access to school records. Thus, the Center for Disease Control and Prevention (CDC) released in April 2018 its biennial update of ASD estimated prevalence among children from the US, based on an analysis of 2014 medical records and, where available, educational records of 8-year-old children from 11 monitoring sites across the United States. The new estimate figure represents an increment of 15% in prevalence nationwide, to 1 in 59 children (from 1 in 68 two years previous). The gender gap in autism has decreased. While males were 4 times more likely to be diagnosed than females (1 in 37 versus 1 in 151) in 2014, the difference was narrower than in 2012, when males were 4.5 times more frequently diagnosed than females. This reveals an improvement in the identification of autistic females. Regarding the racial/ethnic disparities in the diagnosis of ASD in the US, it has also narrowed since 2012, particularly between black and white children. This may reflect increased awareness and screening in minority communities. However, the diagnosis of ASD among Hispanic children still lagged significantly behind that of non-Hispanic children. Disappointingly, the new report found no overall decrease in the age of diagnosis. In 2014, most children were still being diagnosed after age 4, though autism can be reliably diagnosed as early as age 2. Earlier diagnosis becomes crucial since early intervention affords the best opportunity to support healthy development and deliver benefits across the lifespan [35].

As for the European Union, the number of reported cases of autism has increased rapidly in all countries where prevalence studies have been conducted [17, 22, 23, 37, 38, 39]. The average figure indicates that ASD affects around 1 in 100 people in Europe, although some studies have found higher and lower prevalence rates of autism. This is to be expected, given that prevalence studies vary in their scientific method and most are based on a limited sample of a country's population, rather than on national statistics. To increase our understanding of autism and improve responses to the condition, Autism Spectrum Disorders in Europe (ASDEU) [40] has been funded by the European Commission to research autism diagnosis, prevalence and interventions and to improve care and support for people with ASD. This

three-year European Union funded research commenced in February 2015 and is currently making final changes to the report with the Commission. Up to know, the updated findings of ASD prevalence in some European countries have been reported as follows: Denmark, Finland and Iceland estimated the prevalence of ASD in 7-9 year old children using nation-wide registries data. Whilst France will be doing the same but with regional statistics. For these four studies the definition for ASD are ICD10 diagnosis codes: F84.0; F84.1; F84.8 and F84.9. Denmark has an ASD prevalence rate of 12.4 per 1,000 and a total of 2414 cases aged 7-9 in 2015. Iceland has an ASD prevalence rate of 26.8 per 1,000 and a total of 363 diagnosed cases (Live births 2006-2008 with a diagnosis of ASD by 2015). Finland had an overall ASD prevalence rate of 7.7 per 1,000 and a total of 1841 cases. Based on all children born in Finland 2006-2009 and followed up by 31.12.2016. In France 2 regions were researched giving a total number of 517 ASD cases aged, with a prevalence of 5.4 per 1,000. In terms of screening measures that were examined across seven countries Poland, Bulgaria and Spain were all approximately within 50% -66% of finding new cases per assessment. Ireland assessed 10 individual and found 2 new cases so 20%. Whilst Portugal with 6 new cases from 85 assessments and Romania with a 100% diagnosis rate from 20 cases create an anomaly for the researchers. Italy's figures still need to be confirmed [41]. The increase in prevalence estimates may represent changes in the concepts, definitions, service availability, and awareness of ASDs in both the lay and professional public.

1.2.2.2 *The role of risk and protective factors in ASD*

Although epidemiological studies have reported several risk factors [42], none of them have been proven to be essential or sufficient alone for ASD to develop [13]. Understanding the genetic and environmental interplay and how the complex interactions of genes, experiences, epigenetics, and developmental timing give rise to individual differences in behavior in autism is nowadays a relevant topic for research that is still at an early stage [43, 44, 45]. It has been reported that advanced parental reproductive age is linked to increase autism risk, also evident for a combined parental age effect. Thus, risk is highest when both parents

are older, but the risk is also increased between disparately aged parents [46, 47, 48, 49]. The subjacent molecular mechanisms remain unclear, but the leading hypothesis may be related to germline mutation, especially when paternal in origin [50, 51, 52, 53, 54]; a second hypothesis concerns mechanisms related to epigenetic alterations resulting in altered chromatin structure and DNA-methylation patterns [55]. In addition, ASD prevalence has been reported to be two times higher in areas with high rates of employment in the information-technology-engineering sector, indicating that parents of autistic children may be more likely to be technically talented than other parents, having highly technical and structured occupations in fields such as science, engineering, and accounting [13, 56, 57, 58, 59, 60]. In fact, some studies have suggested that parents of children with ASD may actually have a "broader autism phenotype" that is less recognizable and can be magnified in offspring [61]. Prenatal risk factors that may affect neurodevelopment, such as gestational diabetes [62, 63], exposure to chemicals [42, 64, 65, 66, 67, 68] and other complications during pregnancy have been proposed to increment the risk of suffering ASD [69, 70]. Several none-specific groups of disorders affecting perinatal and neonatal health status have been also related with increased autism risk [13, 71, 72]. On the contrary, folic acid supplementation prior to conception and during early pregnancy has been reported to be a protective factor against ASD [13, 73, 74]. Finally, there is no evidence that MMR (measles, mumps and rubella) vaccination [75, 76], thimerosal-containing vaccines [77, 78] or repeated vaccination [79, 80] cause ASD [13].

1.2.2.3 *Comorbidity and ASD*

Nearly three-quarters of children with autism spectrum disorder (ASD) also have another concurrent medical, developmental or psychiatric condition [81, 82, 83, 84, 85, 86], even an increased proportion have been reported for psychiatric outpatients [87] and patients from specialist medical attention [88]. This is called "comorbidity", and the conditions are often called "comorbid" conditions (Figure 1.2). Characterizing the heterogeneity of ASD is further complicated by the occurrence of these comorbidities.

Comorbid psychopathologies significantly over-represented in ASD include anxiety, depression, ADHD, and intellectual disability; and medical comorbidities include seizures, sleep difficulties, gastrointestinal disorders, mitochondrial dysfunction, and immune system abnormalities [88]. The presence of one or more of these comorbidities is likely to be associated with more severe autism-related symptoms. For example, individuals with ASD who also have epilepsy are more likely to have severe social impairments than those diagnosed with ASD only.

Furthermore, sleep problems could exacerbate the severity of core ASD symptoms [89] and aberrant behaviors are correlated with gastrointestinal problems in young children with ASD [90]. The role of immune system abnormalities in ASD is a significant focus of ongoing research. Altered immunity involving cytokines, immunoglobulins, inflammation, cellular activation, and autoimmunity have all been implicated in ASD and altered levels of cytokines have been associated with the severity of behavioral impairments, although the complex nature of these relationships limits the characterization of these associations between comorbidities in general and the severity of autism-related symptoms [91, 92, 93, 94].

Comorbid conditions can appear at any time during a child's development. Childhood co-occurring disorders tend to persist into adolescence [95]. Some might not appear until later in adolescence or adulthood, such as epilepsy or depression [13]. In general, the more concurrent conditions, the greater the individual's disability [84]. Sometimes, these comorbid disorders have symptoms that affect how well ASD therapies and interventions work, so it is crucial to identify the conditions and treat them separately. This high rate of comorbidity may result from a shared pathophysiology, side effects of growing up with autism, common symptom domains and associated mechanisms, or overlapping diagnostic criteria [13]. Improved characterization of autism concurrent conditions is imperative for the development of a comprehensive understanding of ASD heterogeneity and may lead to the identification of distinct subgroups of ASD and subgroup-specific treatments [96].

Conditions	Percentage of autistic individuals affected
Developmental	
Intellectual disability	≈45%
Language disorders	21-76%
Attention Deficit Hyperactivity Disorder	28-44%
Tic disorders	14-38%
Motor abnormality	≤79%
General Medical	
Epilepsy	8-30%
Gastrointestinal problems	9-70%
Immune dysregulation	≤38%
Genetic syndromes	≈5%
Sleep disorders	50-80%
Psychiatric	
Anxiety	42-56%
Depression	12-70%
Obsessive-compulsive disorder	7-24%
Psychotic disorders	12-17%
Substance use disorders	≤16%
Oppositional defiant disorder	16-28%
Eating disorders	4-5%
Personality disorders	
Paranoid personality disorder	0-19%
Schizoid personality disorder	21-26%
Schizotypal personality disorder	2-13%
Borderline personality disorder	0-9%
Obsessive- compulsive personality disorder	19-32%
Avoidant personality disorder	13-25%
Behavioral	
Aggressive behaviors	≤68%
Self-injurious behaviors	≤50%
Pica	≈36%
Suicidal ideation or attempt	11-14%

Figure 1.2: Common ASD Comorbid conditions [13, 15, 16]

1.2.3 Prognosis and long-term outcome

A meta-analysis study reported that autistic individuals have a mortality risk almost three times higher in comparison with unaffected individuals of the same age and gender [97]. This disparity might be mainly related to comorbid medical conditions [98]. The symptomatology of ASD is extensive and pervasive, with a variable onset that could be considered a dimensional process [99]. Previous studies conducted prior to the widespread application of early intervention programs showed that approximately 60-80% of adults with ASD have poor or very poor outcomes in terms on independent living, educational achievement, employment and peer relationships [13, 100, 101, 102].

Although autism is thought as a lifelong condition, we can find different prognoses with the recent identification of an optimal outcome by which children previously diagnosed with an ASD were no longer considered to meet the diagnostic criteria [103]. The discovery of this outcome challenges the premise that ASD phenotypes are stable and insensitive to treatment, suggesting that different developmental trajectories can diverge considerably [104]. Thus, a higher level of childhood intelligence, phrase speech communication before 6 years of age and less social impairment during infancy prognosticate better outcomes [13, 100, 101, 102]. However, even for autistic subjects without intellectual disability, adult social outcome is usually unsatisfactory regarding quality of life and accomplishment of occupational potential [102], even though it is related with cognitive increase and an improvement of adaptive functioning during development [13, 105]. Diverse developmental trajectories in children with ASD and their siblings have been reported by several childhood follow-up studies [106, 107, 108]. The best possible outcome, such as reversal of diagnosis, negligible autistic symptoms and normal social communication, has also been shown [103, 109].

Interestingly, autism symptoms onset has been a focus of research that enabled the identification of an early onset pattern and a regressive onset pattern in which children appear to develop in a typical way before progressively losing skills and start to develop ASD-like features [110]. Having said that, a deeper review of these conceptualizations concluded that

ASD onset, or at least the arise of its symptoms, is better considered a dimensional process and a continuum in which the early onset and regression patterns represent two extremes [109, 111].

Although there is an increasing recognition of the need for young people with ASD to have a planned transition from child to adult health services [112, 113], to date, there has been very limited research on how to address this topic [114, 115]. Funding differences in child and adult services, divergent eligibility criteria and insufficient awareness among physicians or other professionals may result in mismatched resources. Thus, young autistic adults could become lost at healthcare at a crucial time of high vulnerability on transition from teenage to adult health and education systems. Not surprisingly, it may be confusing for professional and families to know how to navigate the healthcare system between child and adult services [116].

Therefore, transition to adulthood is a difficult task, since it frequently means loss of school support and child and adolescent mental health services. Usually, finishing secondary education goes along with slowed improvement, mainly a result of reduced occupational stimulation and not sufficient adult service development [117, 118]. Even though consensus guidelines regarding transitions between child and adult services for young adults with special care needs have been available for the last decade, there remains a lack of clear pathways providing planned and informed transition for autistic individuals. Furthermore, there is an increased recognition from professionals and people affected by ASD of the need for transition services that also consider lifelong functioning and adaptive skills. In spite of this, autistic young people are 64% less likely than other youth with special care needs to receive transition services to adult care [114, 115, 119].

Finally, little is known about the mental and physical health of older adults with ASD and how ageing affects these individuals [120, 121]. The estimated mean proportion of autistic adults in employment (regular, supported or sheltered) or full time education is

approximately 46% [13, 120]. Since an increasing number of adults are being diagnosed with ASD, further research is needed in this field [122].

1.2.4 Neurobiology

Over the last few years, understanding the cognitive processes involved in a condition like autism has been the main purpose of most studies that have been carried out. Even though genetic factors also play an important role in the occurrence of this condition, the diagnosis is still made based on behavioral analysis and examinations. Neurobiological findings have identified brain perfusion patterns, neural biochemical features, systems level connectivity characteristics and possible neuroanatomical, cellular and molecular underpinnings of ASD [13]. Attempts to propose unified theories elucidating core and comorbid impairments in ASD have been fruitless. This is not surprising considering that the heterogeneity in etiology, phenotype and outcome are hallmarks of this condition. However, significant evidence led to various cognitive theories that have been coined to provide a better explanation about this disorder. None of them fully explains ASD, but they support the premise that neural networks in autism are atypical in diverse ways. Here we briefly explain the most relevant [13, 123].

1.2.4.1 *Theory of impeded plasticity*

It is broadly known that the brain of autistic children presents functional and morphological dysfunctions. Several studies have already showed the existence of a significant decrease in long-distance connectivity in the brains of ASD subjects, using functional magnetic resonance imaging [124, 125]. At the microstructural level, it has been reported that disruption of brain development may result from abnormal regulation of cell division and, as well as high neuronal inflammation [126]. Other studies exhibited that hypo and hyper connectivity patterns could be observed in the brain of children with ASD; the difference in both patterns of connectivity may rely on age-related factors [127, 128, 129]. Thus, a study showed that 3 months old children who are at high risk for developing ASD show increased connectivity in

comparison with low-risk children; this difference begins to gradually disappear between the age of 6-9 months old [130]. Finally, there is also some evidence suggesting that the brain of ASD subjects is defined by morphological abnormalities, such as early overgrowth of several brain regions embracing the frontal cortex, the amygdala and the cerebellum [126, 130, 131].

1.2.4.2 *Excitatory-inhibitory balance dysregulation*

The normal function of brain sensory and cognitive networks depends heavily on a well-balanced development of excitatory and inhibitory synapses. Imbalance between excitation and inhibition and increased excitatory-inhibitory ratio might result in the pathogenesis of several neuropsychiatric disorders, including autism. An imbalance in this development may be responsible for the learning and memory, cognitive, sensory, motor deficits and seizures occurring in this condition [132, 133, 134, 135, 136, 137]. Glutamate and gamma-amino butyric acid (GABA) are the major neurotransmitters in the human brain as they work together to control many processes, including the overall level of excitation. In the mature central nervous system, gamma-amino butyric acid (GABA)-interneurons send inhibitory synaptic inputs, while glutamatergic neurons send excitatory inputs. Environmental factors and mutations that increase glutamate signaling or decrease GABAergic signaling may lead to an imbalance of excitation-inhibition, altering the manner by which the brain processes information and regulates behavior, and, therefore, increasing the risk to develop ASD [136, 137]. Thus, the imbalance in autistic individuals is mainly due to abnormal glutamatergic and GABAergic neurotransmission in crucial brain areas such as neocortex, hippocampus, amygdala and cerebellum. Other causes of the aberrant synapses or connection between brain cells in autism appear to be dysfunction of neuropeptides (oxytocin), synaptic proteins (neuroligins) and immune system molecules (cytokines) [135].

Several studies support the leading hypothesis that a generalized hyper-excitability in the brain of individuals with ASD may cause cognitive functions impairment and increase seizure susceptibility. Thus, a significant amount of research suggests that autism patients have higher than normal glutamate blood levels [138, 139, 140, 141, 142, 143]. GABA, which

plays a crucial role in the regulation of neuronal excitability, is also reported to be altered in autistic individuals. Hence, GABAergic neurotransmission alterations in key brain regions of subjects with ASD could explain diverse clinical phenotypes, such as learning disabilities, mental retardation or the development of epileptic seizures [144, 145, 146]. Furthermore, variations in both serotonin and GABA systems have been steadily observed in individuals with autism, like hyperserotonaemia and an alteration of brain serotonin synthesis capacity, and a decreased expression of GABA synthetic enzymes and receptors [147]. In general, multiples lines of evidence implicate a marked dysregulation of inhibitory GABA system and glutamate system in individuals with ASD as a shared pathophysiological mechanism [132, 133, 134, 135, 136, 137]. However, it is not very clear how these synaptic inputs affect neuronal brain circuits and social behavior. Consequently, more exhaustive behavioral and electrophysiological studies need to be conducted to better define the role of excitatory/inhibitory neurotransmission in social and cognitive functions. Finally, understanding the molecular underpinnings of this imbalance may provide essential insights into the etiology of these heterogeneous and complex disorders and may uncover novel targets for better diagnosis and future drug discovery in ASD [148, 149, 150, 151, 152].

The role of oxytocin and arginine-vasopressin systems in ASD social impairments are also an active focus of research in ASD, including clinical trials. There is strong evidence implicating these neuropeptides in complex social behaviors such as social stress, social cognition, trust, social approach, and aggression, and therefore, relating these brain chemicals with the modulation of affiliative and social behavior and cognition, which may play a key role in autism's etiology and development [153, 154, 155, 156, 157].

1.2.4.3 *Theory of mind*

Over the last decades, studies on theory of mind (ToM) have controlled research on autistic individuals and have unveiled significant impairments in integrating mental state information [158, 159, 160, 161, 162]. ToM can be defined as an understanding that others have minds that are different from our own. More specifically, it is the capacity to mentally understand

that others have thoughts, feelings, beliefs, attitudes, desires and perspectives that differ from ours, regardless of whether or not the circumstances are real [163]. The theory of mind phenomenon appears to be unique to children with autism, that have this capacity delayed in time in comparison to non-disabled children who develop it much earlier [163, 164]. Interestingly, autistic individuals have difficulty understanding when others don't know something. It is quite common, especially for those with savant abilities, to become upset when asking a question of a person to which the person does not know the answer. Actually, autistic children usually don't pass standard ToM test, that requires them to comprehend that other people can have different or factually incorrect beliefs about the world [164, 165, 166, 167, 168]. Within the context of autism spectrum disorder (ASD), deficits in ToM may be at the core of many of the behaviors associated with the disorder. Thus, failure to understand that other people think differently than themselves could explain the impairments in social behavior and communication skills reported in autistic individuals. By contrast, adults with ASD generally succeed on first-order ToM tests, although they are still unable to understand other people's cues, beliefs and intentions [164, 165, 166, 167, 168, 169]. Some researchers have also hypothesized a social-affective justification, supporting the premise that a deficit in ToM in autistic individuals results from a distortion in recognizing and responding to emotions [170, 171]. In addition, ToM challenges may lead to limited expression of empathy toward others. ToM deficits may also result in one approaching a social situation with assumptions that may not be accurate. Finally, reciprocity (the give-and-take, mutual benefit of a relationship) may be impacted, as a result of having challenges picking up on cues from the social environment. Thus, the study of social skills in children affected with ASD become crucial for the identification of effective therapeutic treatments and rehabilitation programs that may improve empathic and emotional abilities [170].

1.2.4.4 *Mirror neuron system and ASD*

Mirror neurons are nerve brain cells that are activated when an individual performs and action, but also when observes an action being performed by someone else [172]. These

neurons are believed to be implicated in several functions including cognitive abilities such as learning by imitation, empathy and emotional states, recognition of motor acts by others, and regulation of social tasks [173, 174, 175]. In addition, these neurons also stimulate the coordination between the motor cortex and higher visual processing regions, and are therefore involved in language and speech, memory, planning, reasoning, judgement and voluntary motion [176, 177]. Although there is no reliable neurophysiological marker associated with ASD, there is increasing evidence highlighting the relevance of mirror neurons in the neuropathophysiology of autism [171, 178, 179].

Overall, there is evidence suggesting that a dysfunction in the mirror neuron system may create cognitive, communicative and social impairments in individuals with autism [174, 178, 180, 181, 182]. These deficits are more pronounced when ASD subjects complete tasks with social relevance, or that are emotional in nature. Evidence of these deficits in ASD subjects comes from a diverse array of different neuroscience techniques, such as functional MRI (fMRI), eye tracking, transcranial magnetic stimulation (TMS), electromyography (EMG) and electroencephalography (EEG) [179, 181, 182, 183, 184]. The current data obtained from the conducted research is very mixed, and some results are discordant and difficult to interpret. Thus, fMRI studies that use emotional stimuli as a measure of mirror system are those who have shown significant differences between groups, in comparison with other studies that used non-emotional hand action stimuli [182]. In addition, another study that measured through EMG the activity of the mylohyoid muscle, implicated in the opening movement of the mouth, reported lower EMG activity in ASD children when compared to typically developing children, holding the premise that autistic individuals may exhibit a dysfunctional mirror neuron system that results in cognitive and attention impairments [183].

By contrast, other works illustrated that the functionality of the mirror neurons might be preserved in subjects with autism [185, 186]. For example, EEG mu (μ) rhythm was recorded in individuals suffering from autism and age-matched typically developing subjects while watching an action being performed by an experimenter [185]. Well, results from this

research showed that the activity of mirror neurons during the observation exercise was not related with ASD, but rather with disparities in the ability of imitation, insinuating a clear dissociation between ASD and the mirror neuron system [185]. Such discrepancy between studies may be a consequence of the different methodological approaches employed as well as divergences in the diagnostic criteria used when choosing individuals with ASD [123]. Although the research base is not very large, most of the studies support the hypothesis of impaired mirror neuron system in ASD as promising. Given the relevance of some findings, to continue exploring the link between mirror neuron dysfunction and autism may help elucidate the neural basis of autism and may point the way to early diagnosis and potential therapies for clinical/educational practice [174].

1.2.4.5 *Immune system and ASD*

There is increasing evidence to show a substantial interaction between the immune and the nervous systems throughout life, challenging the theory of the so-called immune privilege of the central nervous system (CNS) [187]. Indeed, several independent studies have suggested a role for the immune system in ASD during both prenatal and postnatal stages, and many researchers focus on further investigations of this link, with particular emphasis in establishing whether the immune abnormalities reported in individuals with ASD are a cause or a consequence of the alterations in neurodevelopment, or merely an epiphenomenon of autism [13, 188, 189, 190].

In ASD, immune dysregulation disturbs a broad group of developmental processes (neurogenesis, proliferation, apoptosis, synaptogenesis and synaptic pruning) with persistent active neuroinflammation, high levels of pro-inflammatory cytokines in serum and cerebrospinal fluid, and altered cellular immune functions [191]. This immune dysfunction alters brain connectivity contributing to ASD pathology. Furthermore, maternal IgG antibodies targeting the fetal brain or other gestational immune system abnormalities may be pathogenic in certain cases [192]. Finally, several epidemiological studies highlighted the role of maternal autoimmune conditions during pregnancy in ASD, reinforcing the notion that ab-

normal maternal immune activation may have adverse effects on fetal brain development [94, 193, 194, 195, 196].

1.2.5 Causes of autism spectrum disorder

Although the exact cause of autism spectrum disorder remains unknown, it is clear that is highly heritable and, given the complexity of the condition and the variety of symptoms and severity, both genetics and environment may play a role in its etiology.

1.2.5.1 *Genetics*

Autism spectrum disorders are persistent and lifelong conditions highly heritable, with both common and rare variants contributing to its etiology, as supported by twin and family studies that raised questions about the relative influence of genetic modifiers [197, 198, 199, 200, 201]. The genetic architecture of autism has proved to be heterogeneous and complex, as exhibited by cytogenetic, linkage, association, whole-genome linkage or association, microarrays and whole-genome or exome sequencing studies [202, 203]. The development and implementation of high-throughput microarray and sequencing platforms have made possible major advances in our understanding of autism genetic risks factors.

Many genetic variants associated to ASD present a high degree of pleiotropy, as well as a high rate of locus heterogeneity. Both rare mutations with large effect sizes and common variations with smaller effect sizes have a role in the genetic etiology of autism [203]. Rare mutations are regularly detected in ASD and may occur in the form of the so-called syndromic autism (it arises in approximately 5-10% of ASD individuals with co-occurring neurological monogenic disorders, such as Fragile X Mental Retardation, Tuberous Sclerosis or Rett Syndrome, and harbor a set of phenotypes that can be fully attributed to a mutation in a particular gene or a set of genes [204]), chromosomal abnormalities, rare copy number variations (CNVs) and single nucleotide variants that have been identified by exome sequencing and single-nucleotide polymorphism (SNP) arrays [201, 205, 206]. The most recurrent

chromosomal abnormality is maternally derived 15q11q13 with variable size, appearing in 1-3% of affected individuals. Many genes of this chromosomal region (GABRA5, GABRB3, UBE3A, HERC2, SNRP and CYFIP1) have been reported to be involved in crucial functions in the brain, such as neural differentiation, growth, development, maintenance of function and circuit organization [207].

De novo mutations, such as copy number variations (microdeletions or microduplications), and single nucleotide variants (nonsense, splice-site and frameshift mutations) which took place in the germline (particularly paternal) have been reported to have a large effect size and could be causative, especially in simplex families where only one individual has been diagnosed with ASD [13, 52, 53, 54]. Similarly, copy number variations with medium effect sizes and variable expressivity and penetrance are likely to have some role [203]. Nevertheless, each identified copy number variation only takes place at the most in about 1% of autistic individuals, suggesting again the presence of significant genetic heterogeneity in these disorders [203]. The most common recurrent CNVs linked to ASD are microdeletions and microduplications, particularly the 16p11.2 deletion has been associated with the risk of morbid obesity and macrocephaly, while the 16p11.2 duplication has been associated with low body mass index (BMI) and microcephaly [208]. Other frequent CNVs identified in ASD patients include 1q21.1, 15q13.3, 17p11.2, 22q11.2, 16p13.1 and microduplication of 7q11.23. Moreover, microanalysis revealed several non-recurrent microdeletions including regions of 2p16.3, 7q22q31, 22q13.3 and Xp22 [207].

With regard to common variants, recent genome wide association studies (GWAS) have detected some relevant single nucleotide polymorphysms, but none of them present a large enough effect to be considered causative or at least to have a genome-wide level of significance [202, 209]. In spite of these discouraging results, these and other GWAS revealed an important contribution of common variants in autism's inheritance. In addition, the more complex the disorder is, the greater the probability that many genes are linked and many different polymorphisms affect its heterogeneity, thus much greater cohorts must be tested [207]. Furthermore, this contribution remains the same irrespective of whether or not

there is a background of de novo mutations; in fact, contributions from rare and common genetic variants are not only not mutually exclusive, but rather could have an additive effect to create risk for ASD [210]. Mutations and copy number variations affect genes encoding for proteins with a crucial role in the chromatin remodeling (CHD8, BAF155), as well as synaptic cell adhesion molecules (Neurexin and Neurologin families, CNTN4), neurotransmitters, scaffolding proteins in synapse (SHANK2 and SHANK3) and ion channel proteins (CACNA1A, CACNA1H, SCN1A, SCN2A). The proteins are implicated as well in the signaling pathways and neuronal networks related to synaptic gene transcription and translation pathway (FMR1, TSC1, TSC2, PTEN, NF1, CYF1P1), ubiquitination pathway (UBE3A, PARK2, TRIM33), protein synthesis and degradation, and are also enrolled in the creation, development and function of synapses and neurons. Thus, the existence of an imbalance in the excitatory-inhibitory synapses could be the reason for the deficit in social behaviors and cognitive functions present in autistic individuals [211, 212, 213]. Moreover, a study reported that genes involved in synapse formation and function exhibit a significantly lower expression in autistic brain when compared to the normal brain, providing strong evidence for the implication of transcriptional and splicing dysregulation as underlying mechanisms of neuronal dysfunction in this disorder [214].

1.2.5.2 *Environment*

Although ASD has been shown to be highly heritable, current evidence suggest that this condition cannot be solely explained by genetics. In fact, novel technologies and epidemiological studies have reported multiple correlations between non genetic influences and autism, providing new insights into the possible role of environmental factors in the etiology of ASD and leading the way for further research to investigate mechanisms and establish its cause [201, 215].

Maternal treatment during the first trimester of pregnancy with certain drugs, such as valproic acid, thalidomide and some type of antidepressants, has been related with an increased risk of ASD in the offspring. In addition, pregnancy-related complications and conditions,

metabolic and nutritional risk factors, such as diabetes, obesity, folic acid deficiency and maternal bacterial and genitourinary infection, and toxic habits like smoking and alcoholism, include a small but significant high risk of ASD [216]. Exposure to toxins including pesticides, heavy metals, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), ozone, diesel, small particulate matter, thimerosal and inorganic mercury may have harmful consequences on developmental processes, particularly for genetically susceptible individuals. Indeed, these neurotoxic compounds may interfere with neurotransmitter systems also linked to autism [190].

Advanced parental age (in both mothers and fathers) has also been well established as a risk factor of ASD in the offspring, as commented before. In fact, it is thought that it contributes to methylation defects in gametes, causing DNA damage and fragmentation through increased oxidative stress [190, 215].

Despite intensive research, it seems that no specific prenatal, perinatal and postnatal risk factor has been consistently validated as an independent environmental risk factor for autism. Since humans are continuously introduced to several external environmental agents that may have an adverse impact on fetal development, it becomes essential to promote preventative measures and further research [215, 216].

1.2.5.3 *Gene-environment interplay*

Nowadays, it has been widely accepted that the majority of disorders result from a complex interaction between an individual's genetic profile and the environment that he is exposed to. Thus, although autism susceptibility is currently estimated to be 40-80% genetic, environmental factors, frequently acting through epigenetic regulation as the main mechanism, presumably contribute to an increasing risk of this condition. Environmental factors, like the ones described above, may directly act with some susceptibility genes, resulting in epigenetic changes in gene expression that facilitate the development of ASD [201]. Evidently, epigenetics could have a deep impact on the transcriptome of an individual. Pathogenic

variants in one epigenetic-regulating gene or even effects from the environment may widely spread gene dysregulation. Epigenetic modulators may not only be themselves causative of a pathogenic condition, but also may exacerbate or ameliorate the disorder phenotype by influencing expression of risk genes [201].

Despite the vast evidence for the involvement of gene-environment interaction in the etiology of ASD, it is worth mentioning that the impact of genetic and environmental factors varies according to cases. In fact, because ASD is such a complex and heterogeneous disorder, no single or major environmental factor has been identified to date. More studies are needed to better understand the common ASD epigenome and whether certain epigenetic markers might be protective or detrimental depending on the individual's susceptibility. In addition, it becomes crucial to decipher epigenetics as a link between ASD genetic predisposition and environmental risk factors. Future research should be focused on a combination of factors through an integrated approach that embraces gene-environment interactions and multivariate analyses, shedding light into potential therapies [201, 215, 216].

1.2.6 Diagnosis and therapeutic intervention of autism spectrum disorder

Over the past decades, a range of screening and diagnostic instruments for ASD has been developed and widely validated for use either in the clinical practice or the research field [14, 217]. They differ in several aspects and vary from checklist questionnaires for screening and rapid ascertainments of symptom severity to structured tools, including the Autism Diagnostic Interview, Revised (ADI-R) [218], the Developmental, Dimensional and Diagnostic Interview (3di) [219] and observational measures such as the Autism Diagnostic Observation Schedule, second edition (ADOS-2) [220], among others [14, 217]. However, they present some limitations, being the most notable the degree to which the accuracy and effectiveness of these instruments has been validated in autistic individuals [14, 217].

The neurological alterations leading to autism likely occur during pregnancy and both biological and behavioral evidence show that something is going wrong within the first year of life (or earlier), although the disorder is almost always clinically undetected at that age [221]. Despite huge efforts for early identification of the condition, the mean age of diagnosis is still not made before a mean age of 3 years [217]. That is because ASD remains a behaviorally defined disorder, placing limits on the age at which a confident diagnosis can be made [221].

However, our understanding of autism spectrum disorders has substantially changed since it was first described by Kanner [11]. Assessment of ASD is a slowly changing landscape that impacts the lifespan and is not merely making a one-time diagnosis; in fact, the needs of autistic individuals change as they develop over time, thus, requiring monitoring and re-evaluation throughout life [217].

As behavioral measures continue to be used for the early detection of ASD, experimental technologies are also proving effective in detecting the first signs of this condition, some even before behavioral features are clinically observed [217]. Thus, neuroscientists are serving to elucidate the development of autism over time. To date, neuroimaging studies have reported brain abnormalities such as atypical brain morphology and aberrant brain activity during a critical age development period, when the formation and connectivity of cerebral circuits are in the most productive and optimum stage of synaptic activity [222]. Similarly, other studies regarding eye-tracking behaviors and joint attention skills have also indicated that high risked ASD infants showed delayed responses when shifting eye gaze during tasks measure eye movements and eye contact and exhibited atypical patterns of visual preference when compared to their neurotypical peers [217, 222].

Recent discoveries about autism's etiopathogenesis, symptom structure and heterogeneity are challenging traditional nosology and driving efforts to reconceptualise the diagnostic boundaries of ASD in order to shift the current paradigm. Next generation advances in the classification of this condition could be related to several improvements in the diagnosis of ASD that may have an important effect on clinical practice, research, public health and

policy [14]. Multidisciplinary scientists and an exponential growth of ASD research have made possible key achievements, such as: reaching a consensus about behavioral definition; accepting the increased prevalence of this disorder; improving the understanding about early screening and diagnosis; establishing systematic clinical assessment measures and evidence-based interventions; clarifying specific cognitive processes; and employing a systems-level approach to understand neurobiology [13, 14, 217].

Notwithstanding these efforts, there are still limited treatment options to ameliorate the symptoms related to this disorder, including those symptoms derived from comorbid mental and medical conditions that may exacerbate the severity of presentation. Although there are promising signs for new autism medical treatments, recent reviews suggest that there is minimal evidence supporting the benefit of most treatments in the cases of autistic children receiving therapeutic interventions [15, 223].

Progress in systems biology interdisciplinary approaches, embracing various fields of study such as developmental biology, genetics, epidemiology, neuroscience, biochemistry, statistics, mathematics and computational biology, among others, will ultimately help to improve the diagnostic process and enable the identification of biomarkers and different subtypes of autistic individuals who are likely to benefit from personalized therapies that have yet to be discovered and might provide a basis for ASD early detection and intervention [224].

1.2.7 Systems Biology approaches to understand autism spectrum disorder

As said before, ASD is currently known to be caused by the combination of genetic, environmental, immunological and neurological factors. Thus, it becomes necessary to address the study of the complexity and heterogeneity of this condition from a novel perspective. The development of high-throughput technologies represents an enormous challenge for researchers, due to the large amount of high dimensional data generated. All this biological information must be analyzed from an integrative perspective that attempts to understand

higher-level operating principles of biological processes. This is the reason why systems biology has emerged to cope with this complexity and catalyze important changes in the future of healthcare by applying computational methods at multiple levels to these large datasets. Systems biology approaches applied to healthcare would try to identify the systems that, when altered, shift the body from a "healthy" to a "disease" state. The working hypothesis is that the elements of the biological system that are involved in the observed switch between states are specific to the disorder under study, ASD in our case, and may be candidate targets for treatment to restore the system to its original healthy state.

From a medical point of view, systems biology provides a primeval contribution to conduct functional analysis of genomic events that could be widely used in gene finding, biomarker identification, detection of early dysfunctional behaviors, disorder subgroups classification, drug discovery, therapy strategies and, in the last instance, predictive, preventive and personalized medicine [225, 226]. Discerning the causal agents of ASD becomes crucial for effective detection and for finding the most adequate therapeutic intervention. Traditional approaches were pointed towards single molecules or signaling pathways when identifying diagnostic biomarkers. Instead, systems biology strategies focus on the global analysis of multiple interactions at different levels. A differentiated biological function is rarely regulated by a single molecule. Rather, the true nature of biological processes is far more complicated, and most biological features are determined by complex interactions among a cell's distinct components.

For this reason, systems biology strategies usually employ networks as a representation of these biological relationships, enabling to take advantage of the mathematical tools from Graph Theory. Thus, groups of interacting molecules that regulate a discrete function construct bio-modules whose interrelations bring out networks. In the network representation, nodes symbolize the constituents of the system (genes, proteins, enzymes) and links connecting nodes represent interactions or reactions in which these molecules participate. Complex networks algorithms have been developed to visualize, analyze and model curated pathway datasets, integrate networks and perform functional annotations on them [225].

The complexity of ASD points to the dysregulation of not one, but multiple pathways and biological processes. Therefore, network studies attempt to be useful in refining our understanding of the molecular basis of ASD, as they provide valuable information about the dynamics of the underlying pathogenetic events of the clinical phenotype.

There are several functional annotation tools to assist in extracting meaningful knowledge captured by the biological datasets and candidate gene lists derived from network analysis. Briefly, the most widely employed: (a) DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics resources [227, 228] allow gene annotation enrichment analysis, functional annotation clustering and gene functional classification, providing functionally related groups of genes that help unravel the biological content gathered by high throughput technologies; (b) Gene Ontology [229], a significant bioinformatics initiative that standardizes the representation of genes and gene products by developing three organizing principles, describing them in terms of their associated biological processes, cellular components and biological functions in a species-independent manner across multiple databases; (c) Ingenuity Pathway Analysis (IPA) [230], a web-based application for modeling, analyzing, understanding and accurately interpreting complex biological and chemical meaning from genomic data [215]; KEGG (Kyoto Encyclopedia of Genes and Genomes) [231], a database resource for understanding high-level functions and utilities of biological systems, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies.

In summary, systems biology emerges as a set of new analytical techniques developed to harness and tame the complexity of biological interactions. Autism research findings need to be mined, integrated and modeled to help not just future generations, but also to improve the outcomes for the current generation of people with ASD [20].

1.3 Research Aim

The enormous clinical and etiological variability between individuals on the autism spectrum and the lack of effective drugs have made precision medicine the most promising treatment approach. Thus, the identification of novel candidate ASD-risk genes becomes crucial to discover targets in order to develop better diagnostic tools and find effective therapeutic interventions for autistic patients based on these new possible biomarkers. The significance of these discoveries lies in their potential ability to identify a causal link from a gene to cellular and molecular mechanisms underlying ASD symptoms.

Therefore, this Doctoral Thesis aims to take advantage of the advent of high-throughput platforms and the availability of large-scale data sets to perform an integrated systems biology analysis of autism spectrum disorder, with the goal of discovering new candidate genes that may help refine our understanding of its complex genetic architecture, underlying neurological mechanisms and relevant biological pathways involved in the onset and maintenance of this multisystem and heterogeneous condition.

1.4 Objectives

From a research perspective, in this Doctoral Thesis we have conducted different systems biology approaches, including a comprehensive study of brain and blood independent gene expression microarray experiments, a cross-disorder comparative analysis between autism and closely related conditions with an elevated level of co-occurrence with ASD, specially focused on biological processes and gene networks, and a Genomic Evolutionary Rate Profiling (GERP) analysis, with the aim of achieving the following specific objectives:

- To determine whether a common signature representative of ASD exists and to test whether regulatory patterns in the brain relevant to autism can also be detected in blood.

- To identify genes uniquely related to ASD that will allow us to better analyze gene functions and reveal complex disorder genes by describing metabolic and regulatory pathways unique to this condition.

- To find an evolutionary signature intrinsic to the sequences unique to ASD, in order to determine whether the sequence of these characterized genes have been subjected to differential evolutionary constraints, either purifying or positive selection during the evolutionary timeframe for mammals or in the more recent evolution of humans.

- To detect crucial candidate genes in the overlap between ASD and several related concurrent disorders (behaviorally related, comorbid or both) that may help us to decipher common molecular mechanisms and/or a shared pathophysiology and, ultimately, yield powerful insights in the understanding of autism spectrum disorder etiology.

1.5 Results and Discussion

The core of this report consists of three publications through which the objectives set out in the proposed Doctoral Thesis have been addressed and developed.

As explained thoroughly in the previous sections of this report, it has become evident that autism has a complex genetic architecture and, therefore, multiple phenotypes where ASD individuals cluster differently according to widely variations in genetics, clinical presentation, severity of symptoms, comorbidities and treatment response. Although heritability estimates indicate a strong genetic influence in this condition's etiology, no reliable genetic biomarkers for the disorder are available yet. This makes molecular diagnosis and patient prognosis challenging tasks for researchers. Improving early diagnosis and prognosis of ASD using biomarkers with a robust predictive power would benefit autistic patients, making possible an early specific therapeutic intervention. The exponential and diverse types of genetic interactions, biological processes and molecular mechanisms underlying the etiology of ASD evidence the need of a systems biology perspective to embrace this challenge.

Thus, in this Doctoral Thesis, a wide range of systems biology approaches have been applied to the analysis of massive gene datasets obtained from different and independent public sources with the goal of identifying molecular bases subjacent to this condition, such as:

- A molecular signature specific of ASD, detectable both in blood and brain tissue, that may be of potential relevance in the discovery of a diagnostic biomarker that helps to achieve earlier diagnosis and predict clinical prognosis.

- A molecular signature in relation to ASD comorbidities in order to determine whether differential evolutionary constraints and purifying selection in genes unique to autism could be indicators of involvement in recent human evolution.

- A molecular signature related with sibling conditions to ASD with the purpose of detecting biomarkers of genotype-phenotype relationships useful in the development of personalized treatment for autistic individuals.

The following sub-sections are a summary of the different works developed and covered by the proposed Doctoral Thesis, as well as a brief discussion on the results obtained and published in each of them.

1.5.1 A common molecular signature in ASD gene expression: following Root 66 to autism

During the development of this Doctoral Thesis the first task addressed was to conduct an integrated systems biology analysis by comparing a large set of published and openly available gene expression experiments from different tissue types performed in autistic individuals. In this work, the goal corresponds to the first objective described in section 1.4 of this report, that is, to determine whether a common molecular ASD signature exists and to test whether regulatory patterns in the brain relevant to autism can also be detected in blood.

In this study, we performed a distance based clustering analysis on a binary matrix of gene presence-absence obtained from ASD gene expression experiments of 27 case-controls biosets. A statistically significant and highly correlated regulatory pattern of 66 genes was found grouping together involving both, blood and brain tissue, the subset of genes supporting this cluster was named Root 66. Four Root 66 genes (MAP1LC3B, PDE4B, TCF4 and UPF2) were already associated to ASD at that time, 32 had direct interactions with known autism candidate genes and 19 were directly involved in or had links with related neurological conditions, such as schizophrenia, epilepsy, intellectual disability, seizures, attention deficit and disrupted behavior disorders, Angelman syndrome, bipolar disorder, mental retardation, developmental disabilities, sleep disorders and Alzheimer's disease, among others. Furthermore, triangulating with relevant published exome-sequencing studies, 9 of the Root 66 genes have had variants reported as de novo or elevated risk for autism [2, 52, 53, 54, 227, 232].

In order to determine Root 66 gene expression in non-autism disease and normal tissue, the list of differentially expressed genes obtained from 450 disease versus normal RNA expression independent experiments was examined for overlap with Root 66 genes. The outcome was a mean overlap of 6 genes, what strongly supported the hypothesis that the brain-blood cluster generated in our analysis, formed by the Root 66 genes, was unlikely to be a random event and may in fact represent a pattern unique to autism. To further test whether the brain-blood cluster detected in our research could form by chance, results from 5 gene expression studies in normal brain and blood samples were explored [233, 234, 235, 236, 237]; their research confirmed that even though there was overlap in the genes expressed in normal brain and blood tissue, the different tissue transcriptomes analyzed in these studies clustered independently from one another in all cases, unlike what it was observed in our clustering analysis. Additionally, in this work it was also tested whether the genes in Root 66 could play a more generic role as housekeeping genes. Only 3 of the genes conforming Root 66 group overlapped with an experimentally confirmed list of 408 housekeeping genes manually curated from public databases [238].

Since these results lent additional support to the hypothesis that the Root 66 cluster revealed in our analysis was non-random and likely played an important role particular to ASD, another necessary step was to better understand the biological significance of these genes by testing their functional enrichment in specific biological processes and whether they conformed an interconnected biological network. The analysis conducted showed that a substantial number of Root 66 genes were interacting in three relevant biological networks linked to neurological disease in different but significant ways.

Thus, the first network was enriched in biological processes related to brain growth and development that may affect learning and memory, since Root 66 genes were found to be linked to relevant nodes in the network, such as PI3K and NFKB complexes, involved in synaptic plasticity; this network also showed patterns of dysregulation in neuroendocrine activity, specifically an interesting connection between Root 66 genes and endocrine hormones of the hypothalamic-pituitary-gonadal axis, such as follicle-stimulating hormone, luteinizing hormone and gonadotropin-releasing hormone. Even though the principal role of this axis is to control development, reproduction and aging, it is well known that these hormones affect behavior, since they have been reported to modify brain structure and functioning, including dysregulation of the follicle-stimulating hormone, with key roles in brain development and neuronal differentiation [239, 240, 241]. Furthermore, oxytocin, a neurohypophysial hormone that acts as a neurotransmitter in brain, stimulates the activity of gonadotropin-releasing hormone neurons, and, hence, the regulation of gonadotropin release in blood. In fact, oxytocin has been shown to be involved in social behavior, emotion recognition and bonding, along with the establishment of trust among people and social attachment [242, 243, 244, 245, 246]. There is also evidence that changes in the neuromodulatory role of oxytocin are related to diverse mental disorders, including ASD [247, 248, 249, 250]. Additionally, oxytocin receptor gene polymorphisms have been associated with ASD risk [251] and the severity of its symptomatology [252]. Finally, some studies [253, 254, 255, 256] have explored that the administration of the neuropeptide oxytocin (OXT) seems to have potential pharmacological value as treatment for targeting the core characteristics of ASD, based

on the premise that induces behavioral enhancements on tasks assessing repetitive behavior, affective speech comprehension (emotional intonations), facial emotion recognition, and social decision making.

The second network generated in our analysis revealed that 14 of the Root 66 genes were interacting with molecules implicated in biological mechanisms responsible for supporting roles in neurodegeneration, such as nervous system inflammation, loss of neurological function and abnormal morphology of brain. Central genes within the network (APP, PTGS2, ERG and YWHAG), linked to Root 66 genes, were involved in biological processes such as amyloid plaque formation, astrocytosis and gliosis, proinflammatory cytokines, oxidative stress, cognitive degeneration and neurological dysfunction typical of neurological diseases. Several studies have shown evidence of neural cell loss and activation of microglia and astrocytes in ASD [257, 258, 259, 260], as well as high levels of APP [261, 262, 263, 264], suggesting that neurodegeneration may have a role in ASD since it underlies the loss of neurological function in children with ASD who have experienced regression and loss of previously acquired skills and abilities. Alterations in common neurological mechanisms, such as disruption during synaptogenesis may link autism with other brain disorders, including schizophrenia, epilepsy, Alzheimer's disease and Parkinson's disease. Research has shown evidence for impaired neural synchrony and neurotransmission systems as pathophysiological processes implicated in the onset and/or maintenance of these neurological conditions [265, 266, 267, 268, 269, 270, 271, 272].

The third network obtained from the analysis englobed 13 Root 66 genes. The three most connected nodes within the network are genes known to be involved in cancer (TP53, PTEN and AGTR1) and it was enriched in biological processes such as neurodegeneration, abnormal morphology and damage of the nervous system, likely caused by tumorigenesis. Several studies have reported mutations in tumor suppressor gene PTEN found in subgroups of autistic individuals with comorbidities like macrocephaly and/or epilepsy [273, 274, 275, 276]. Additionally, there is evidence suggesting that PTEN mutations may have downstream effects on other ASD gene candidates, perhaps playing a role in the autistic phenotype

[277, 278]. Further research has shown that interactions between defective PTEN and TP53 cause a decrease in the energy production of neurons, which leads to stress and induces alterations in mitochondrial DNA and abnormal levels of energy production in brain areas relevant for social behavior and cognition [279, 280].

While the three networks explained above showed potentially independent roles of small sets of genes contained within the Root 66 group, the global connectivity among member genes of Root 66 was also explored. Thus, by merging these three networks, a single global gene network was generated, showing connections between 42 genes of the Root 66 subset and revealing direct links between these Root 66 genes and genes involved in biological pathways associated to neurological processes like synaptic transmission, neurodegeneration, abnormal brain morphology, learning and memory. In this global network, several ASD-related conditions and functions were statistically enriched. Thus, we may have found evidence to support a key role of Root 66 genes in neurological impairment that may underlie the etiology of autism and could be of potential use as a diagnostic biomarker, although more testing will be required to confirm its biological significance and scope.

1.5.2 Comorbid analysis of genes associated with autism spectrum disorder reveals differential evolutionary constraints

The second task addressed in this Doctoral Thesis was to conduct a cross-disorder analysis of genes associated to ASD to reveal a molecular hallmark related to autism comorbidities and define evolutionary trends of autism genes that could also be related to its molecular pathology. Hence, this work aimed to provide a response to the second and third objectives described in section 1.4 of this report: a) to identify genes uniquely related to autism to better characterize gene functions and detect complex disorder genes by describing significant biological pathways specific for ASD and b) to discover an evolutionary signature inherent to the sequences specific to autism, to determine whether the sequence of these genes have been subjected to differential evolutionary constraints.

For this purpose, a solid literature review allowed us to consolidate 31 comorbid conditions to ASD, and published literature mining tools [281, 282] were used to extract associated genes for ASD and its concurrent disorders. Our search found 1031 genes linked to ASD and at least one other related comorbidity, and a core set of 262 genes unique to autism. Twenty one of the genes specific to ASD overlapped with the highest priority candidates reported in [211, 212], research focused on the analysis of high coverage exome data from 3871 and 2517 autism families, respectively.

The identification of genes uniquely related to ASD allowed us to distinguish metabolic and regulatory pathways putatively specific to this disorder. Genes known to be involved in autism as well as in other concurrent condition covered pathways related to synaptic functions, specifically synapse retrograde signaling, and neural function, such as neurotrophins signaling pathway and the hippocampal long-term potentiation (LTP) pathway, which constitutes the molecular basis for learning and memory [283]. Three hormonal pathways related with ASD have been observed in our functional analysis: the estrogen signaling pathway, for which beta-receptors were reported to be disturbed in autistic individuals [284, 285], the ovarian steroidogenesis pathway [286, 287] and Gonadotropin-releasing hormone (GnRH) secretion pathway, which acts upon its receptor to release the gonadotropins and by cascade reaction affects mitogen-activated protein kinases (MAPKs) pathways [288, 289]. Additionally, two relevant pathways that mediate cell responses were also found, the VEGF signaling pathway, that plays a major role in vascular permeability and has known effects on neuron development [290], and the gap junction pathway, which contains intercellular channels that make possible the direct communication between the cytosolic compartments of adjacent cells [291]. Finally, a pathway related to amino acid production was also uniquely associated with ASD based on our gene sets [292].

Regarding the functional analysis of the 262 genes only involved in ASD, 13 biological pathways were identified as unique to autism, which means they were not implicated in any other comorbid condition. Among these pathways, Mucin type O-Glycan biosynthesis showed several genes linked to ASD; mucins interact with alpha-Neurexins, a type

of presynaptic cell surface molecule essential for neurotransmission and linked to neurodevelopmental disorders such as autism and schizophrenia [293, 294]. Another interesting pathway that appeared to be enriched among the genes specific to ASD is the Sphingolipid biosynthesis, since it has a relevant role in maintaining membrane fluidity and the integrity of lipid rafts, and therefore in brain development, and Glycerophospholipid metabolism, which is also the case for the biosynthesis of unsaturated fatty acids pathway [295, 296]. The Glycosaminoglycan pathway has already been suggested as a biomarker for autism as it may be implicated in the etiology of this condition, with an aberrant extracellular matrix glycosaminoglycan function localized to the sub-ventricular zone of the lateral ventricles observed in autistic individuals [297]. The N-Glycan biosynthesis serves several functions for proper central nervous system development and function. Previous experimental and clinical studies have shown the importance of proper glycoprotein sialylation in synaptic function in ASD [298, 299]. Finally, generic functions already linked to autism, such as basal transcriptions factors, homologous recombination, ribosome and spliceosome were also noticed in our analysis [300, 301, 302]. Several amino acids, particularly lysine, have also been related with ASD when examining plasma levels [303, 304], this is also the case for the co-enzyme Q, from the ubiquinone biosynthesis pathway [305].

Network analysis of genes involved in ASD only was also performed, since interconnected genes unique to autism are of high interest, as any variation in their coding sequence would impact the function of the pathways detected. Our analysis showed that the node with highest connectivity was the gene PIK3R, which is part of the regulatory subunit of a phosphoinositide-3-kinase (PI3K). Activated by many types of cellular stimuli, this gene regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival and, therefore, may play a role in ASD [306, 307]. Genes MAP2K2 and MEK2 were also among the most connected nodes, not surprising taking into account that MAPK (mitogen-activated protein kinase) signal transduction pathway is one of the most widespread mechanisms of cellular regulation known to be associated to ASD [288, 289, 308, 309].

To address the second objective set out for this work, that is, to determine whether the regions of genes implicated in ASD only have been subjected to purifying selection and are enriched for functional elements, in contrast to genes shared by ASD and other comorbid conditions, a Genomic Evolutionary Rate Profiling (GERP) analysis was conducted to compare orthologous genomic DNA sequences. The results indicated that the genes associated to several comorbid disorders seemed to have undergone more purifying selection than those that are unique to autism. This suggests that the biological pathways underlying ASD only (all related to basal cellular roles) were functionally distinct, and may have evolved (or be evolving) under different evolutionary constraints than genes shared between autism and concurrent conditions (found to be associated with neurological functions). Research has shown that coding sequences expressed in the brain evolved at a slower rate than in the rest of the genome [310, 311], which is consistent with our observation that ASD comorbid associated genes are under higher constraints than genes unique to autism, and are also involved in pathways expressed in other regions than the brain. Furthermore, our finding that unique genes to autism have reduced purifying selection in comparison with those genes in the overlap between autism and its comorbid conditions is consistent with other studies [202, 312, 313]. Thus, autism unique genes may experience higher rates of mutation related to cognitive changes observed in human recent evolution.

1.5.3 Cross-disorder comparative analysis of comorbid conditions reveals novel autism candidate genes

Finally, the third task addressed in this Doctoral Thesis aimed to fulfill the fourth and last objective established in section 1.4, that is, to identify key novel autism candidate genes present in the overlap between ASD and its most related comorbid disorders that may enlighten the understanding of the etiology of autism by deciphering common molecular mechanisms and/or shared pathophysiology involved in these conditions.

In order to quantify the existing overlap at the level of molecular physiology and thus, achieve this molecular signature of potential genotype-phenotype relationships, a cross-disorder comparative analysis of ASD and its comorbid conditions was conducted using a two-fold systems biology strategy. As in the previous work, a defined set of 31 comorbid disorders to autism were consolidated from the bibliome [81, 88, 212, 313, 314, 315, 316]. Published literature mining tools [281, 282] were used to generate lists of genes associated to each comorbid condition, and, in the case of ASD, its gene list was further completed by adding the autism candidate genes included in [211, 212, 317]. The retrieved gene lists for each condition were converted into a binary matrix of gene presence-absence in order to generate a disorder phylogeny that helped us identify the comorbid disorders most closely related to ASD. The tree obtained grouped autism with 13 disorders that we called "sibling" comorbid disorders of ASD, which included epilepsy, intellectual disability, fragile X syndrome, schizophrenia, depressive disorder, bipolar disorder and attention deficit hyperactivity disorder (ADHD), among others. Cluster wise validity and stability within the phylogeny was assessed by means of a non-parametric bootstrap procedure that yielded statistically robust results for all clusters including this sibling group, which was considered the focus for subsequent analysis. Gene networks for each member of the ASD sibling group were generated to explore genetic overlap among these conditions. Thus, of the 1066 genes conforming our ASD seed list, 710 had also been related to at least one other autism sibling disorder. This set of genes was called the Multi Disorder Autism Gene Set (MDAG) and constituted a highly interconnected subcomponent of the ASD gene network, suggesting common molecular mechanisms and shared biological functions among the MDAG members. To verify this hypothesis, significant enrichment of MDAG genes in biological processes was identified, revealing relevant and informative over-representation in synaptic transmission, neuron development, axonogenesis, transmission of nerve impulse and learning or memory, among others.

Motivated by these findings, two analytical approaches were devised to test whether information from comorbid disorders could yield meaningful focus for the genome-wide search

for ASD gene candidates. Our first approach was a biological process-driven search for novel ASD candidates based on the mainstream conception that biological processes for which the MDAG genes were enriched are generally relevant for neurological dysfunction. It is further predicated on the assumption that genes involved in these processes that have been tied to one or more ASD sibling conditions, but still have not yet been linked to autism, should be ASD gene candidates. To address this premise, the gene lists of all the ASD sibling disorders were mined to identify and retrieve a non-redundant set of 1588 process-based candidates (PBC). 34 processes were not found among the genes in the autism sibling disorders; all other enriched processes returned 2 or more predictions all of which were linked to at least 2 ASD sibling conditions, but not found in our original seed list for autism. In order to empirically test the relevance of our PBC, available whole-genomic expression data analysis from three independent experiments were used to check whether the PBC were significantly differentially regulated in autistic individuals versus healthy controls. As a result, 80 genes from our PBC were found to be under significant differential expression in subjects with autism in the three datasets. The fact that they have been related to neurological dysfunction together with having been implicated in biological processes that seem to play a role in ASD makes these genes appealing new leads that may help elucidate the molecular pathology of autism.

The second approach was a network-driven search for new ASD genes grounded on the premise that protein interaction networks could provide important and sometimes fortuitous leads for disease causative agents, suggesting potential biomarkers or drug targets and shedding light into the biological mechanisms involved [318, 319, 320]. In this strategy, instead of looking at the entire protein interaction network, the surrounding members of the MDAG genes were explored, with the special focus on their first neighbors that were present in the list of autism sibling comorbid conditions, but absent from our seed list for ASD. A number of genes within these candidates had been previously linked to neurological dysfunction. For example, rare genetic variation in SLC1A2, crucial for proper synaptic activation and neurotransmission, has been related with a wide range of neurological conditions including bipolar disorder, schizophrenia and autism [321, 322]. Epigenetic factors such as methylation of the

NR3C1 gene, that plays a key role in the hypothalamic-pituitary-adrenal axis modulation, our primary stress response system, has been implicated in psychopathological disorders like anxiety and depression [323, 324]. Common variants in MAGI2, a synaptic scaffolding molecule with an essential role in synaptic transmission, are known to be associated to epilepsy and cognitive impairment in schizophrenic individuals [325, 326]. Additionally, mutations in CTNND2, a gene strongly implicated in neuronal development, specifically in the formation and maintenance of dendritic spines and synapses, have also been linked to ASD [327].

Furthermore, other candidate genes such as GRIA1, GRIA2, GABBR1, GABRG2, GABRR2, NRG2, NRG3, GRIK1, GRIK4, GRIN3A and GRM3, with functions that comprise formation of synapse, transmission of nerve impulse, behavior, learning or memory, are among families of genes that have been shown to participate in neurological dysfunction jointly impacted in disorders like ASD, schizophrenia and bipolar disorder [328, 329, 330, 331, 332, 333, 334]. Overall, this analysis generated 1794 network-based candidates (NBC), directly connected to a member of the MDAG but not known yet as relevant for autism. As in the first approach, the NBC were validated by testing for significant differential expression in each of the three independent microarray experiments used before; a total of 91 NBC were found differentially regulated in autistic individuals from the three datasets.

Both analytical strategies leveraged the prior knowledge from two distinct sources, biological processes and protein interaction networks, in order to provide focused sets of genes hypothesized to be under differential regulation in autistic individuals. To prioritize novel ASD candidate genes, the two computational approaches were combined to triangulate the definite set of genes independently predicted and verified by both analyses. A total of 64 significant genes conformed the overlap between PBC and NBC across all three experiments. To cut down the size of this gene set, those genes occurring in two or fewer ASD sibling conditions were removed; this criteria was grounded on the assumption that genes with numerous independent associations to our sibling comorbid disorders are more likely to participate in typical neurodevelopmental processes and functions. A final set of 19 ASD

novel candidate genes differentially expressed in autistic subjects from three independent experiments was predicted by both computational strategies (network and process-based), supporting the hypothesis of common molecular mechanisms between autism and other comorbid conditions. In addition, to better understand the biological importance of the final candidate genes, their enrichment in signaling pathways and biological processes was tested, as well as their interconnectivity within a network. The results obtained in this analysis revealed that our predicted genes were involved in four canonical pathways related to brain structure and functioning, neuroinflammation, neurodegeneration, cognition and behavior [335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346]; changes in these signaling pathways may play a crucial role in the pathophysiology of ASD. Moreover, fourteen of these novel candidate genes interact with other molecules forming a network significantly enriched in biological processes participating in normal brain growth and development. Dysregulation of any of these predicted genes may cause important disruptions in these fundamental pathways modifying neural outcomes and affecting cognition, learning and memory, especially since many of them have relationships with genes already linked to ASD (APP, CYP19A1, ESR1, MAPK1, SETD2, SHANK2, and TRPV1).

Furthermore, some of the most connected nodes within the network (ESR1, TP53, AKT1, MAPK1, Pkcs, EGFR and APP) might support neurological pathways involved in neuronal connectivity and synaptic plasticity; thus, dysfunction of these molecular mechanisms has been associated to social and anxiety-related behaviors, mood conditions, cognitive impairment and loss of neurological function, key features observed in several brain conditions, including ASD [261, 262, 263, 264, 289, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358]. As a final point, a remarkable connection was observed between genes linked to autism and other concurrent conditions and Luteinizing hormone (LH), an endocrine hormone of the hypothalamic-pituitary-gonadal axis that acts in synergy with follicle-stimulating hormone (FSH) that participates in brain development and neuron differentiation [359]. Besides, oxytocin, a neurophysial hormone that also acts as a neurotransmitter in brain, controls the regulation of these hormones release in blood, and, as previously commented in this Doctoral The-

sis, plays a crucial role in social behavior, recognition and bonding [242, 243, 244, 245, 246]. Thus, changes of its neuromodulatory activity have been tied to several mental conditions, including ASD [247, 248, 249, 250]. Interestingly, in our previous research described in sections 1.5.1 and 1.5.2, it was also reported dysregulation of the endocrine activity, specifically an interaction between potential ASD novel candidate genes and endocrine hormones of the hypothalamic-pituitary-gonadal axis.

Altogether, these results lend additional support to the premise that prior knowledge leveraged from ASD sibling comorbid disorders may contribute significantly to the progress in the genome wide search for autism candidate genes.

1.6 Conclusions

In the present Doctoral Thesis, different systems biology approaches have been employed to study the complex heterogeneity of autism spectrum disorder from novel integrative perspectives, in order to discover possible candidate genes and to better understand the biological mechanisms underlying this condition, known to be caused by the combination of genetic, environmental, immunological or neurological factors.

First of all, by integrating and analyzing a complete set of published and publically available independent gene expression experiments covering different tissue types, a statistically significant signal between blood and brain supported by a set of 66 genes was found. This particular signature appeared to be non-random and of potential etiologic relevance to autism, since the majority of its members have links to neurological mechanisms participating in normal brain development and function, learning and memory, neurodegeneration, social behavior and cognition and confirmed roles in neurological disease. Although further analyses and experimental validation are necessary to verify our results, these preliminary findings evidence the existence of a detectable signature in the blood of autistic individuals that echoes what might be an important signal of brain dysregulation, and may be considered as a diagnostic biomarker representative of ASD.

Secondly, a cross-disorder analysis of autism and 31 comorbid conditions was performed with the objective of detecting genes specific to ASD and explore their evolutionary history. A core set of 262 genes unique to autism and involved in relevant regulatory pathways and basal cellular functions was identified. By looking into Genomic Evolutionary Rate Profiling analysis we could determine that these genes unique to ASD were under less evolutionary constraints and have reduced purifying selection when compared to those genes shared between autism and its concurrent disorders. Thus, we were able to find an evolutionary signature intrinsic to genes unique to ASD consistent with the premise that these autism specific sequences may suffer higher rates of mutation related to cognitive changes observed in recent human evolution.

Finally, we explored the hypothesis that the high rates of comorbidity associated to ASD suggest the existence of an overlap in both genes and biological processes shared between autism and its sibling conditions. A twofold computational approach was used to conduct a comparative analysis of ASD and 31 comorbid disorders with the goal of gaining leverage from prior knowledge from these related conditions to predict a set of 19 novel autism candidates that were validated through transcriptome expression profiling experiments. This new set of genes may be considered a potential biomarker of phenotype-genotype relationships, as most of them have already been involved in neurological processes crucial for normal brain growth and function, and play critical roles in neurological disease. Future work will be needed to arrange and reorder genes that have been tied to ASD so far, and possibly reveal novel candidates worth investigating for our understanding of the pathophysiology of autism.

Overall, the findings obtained in this Doctoral Thesis confirm the relevance of combining distinct systems biology techniques in the detection of autism risk genes and the understanding of how these might lead to ASD. In our research, many genes with different functions have been revealed as ASD susceptibility genes, we were able to determine that autism unique genes may have been under less evolutionary constraints, and our computational approaches have demonstrated that pathways necessary for normal brain development and functioning are in fact altered in autism spectrum disorders. Hence, to unveil these candidate genes, bio-

logical processes and molecular pathways may have provided valuable insights into autism's etiology and could be of potential use in next generation research studies for the development of future therapeutic treatments, where the identification of specific biomarkers are of increasing importance for the current challenge of personalized medicine.

1.7 List of Abbreviations

ADHD: Attention deficit hyperactivity disorder

ADI-R: Autism diagnostic interview, revised

ADOS-2: Autism diagnostic observation schedule, second edition

AGTR1: Angiotensin II Receptor Type 1

AKT1: Serine-Threonine Protein Kinase 1

APP: Amyloid Beta Precursor Protein

ASD: Autism spectrum disorder

ASDEU: Autism spectrum disorder in Europe

BAF155: SWI/SNF complex 155 kDa subunit

BMI: Body mass index

CACNA1A: Calcium voltage-gated channel subunit alpha1 A

CACNA1H: Calcium voltage-gated channel subunit alpha1 H

CDC: Center for disease control and prevention

CHD8: Chromodomain-helicase-DNA-binding protein 8

CNS: Central nervous system

CNTN4: Contactin 4

CNVs: Copy number variations

CTNND2: Catenin delta-2

CYFIP1: Cytoplasmic FMR1 Interacting Protein 1

CYP19A1: Cytochrome P450 Family 19 Subfamily A Member 1

DAVID: Database for annotation, visualization and integrated discovery

DNA: Deoxyribonucleic acid

DSM: Diagnostic manual of mental disorders

EEG: Electroencephalography

EGFR: Epidermal growth factor receptor

EMG: Electromyography

ERG: Ets-related gene

ESR1: Estrogen Receptor 1

FMR1: Fragile X mental retardation 1

fMRI: Functional magnetic resonance imaging

FSH: Follicle-stimulating hormone

GABA: Gamma- amino butyric acid

GABBR1: Gamma-aminobutyric acid (GABA) B receptor 1

GABRA5: Gamma-aminobutyric acid type A Receptor subunit alpha5

GABRB3: Gamma-aminobutyric acid type A receptor beta3 subunit

GABRG2: Gamma-aminobutyric acid receptor subunit gamma-2

GABRR2: Gamma-aminobutyric acid receptor subunit rho-2

GERP: Genomic evolutionary rate profiling

GnRH: Gonadotropin-releasing hormone

GRIA1: Glutamate Ionotropic Receptor AMPA Type Subunit 1

GRIA2: Glutamate Ionotropic Receptor AMPA Type Subunit 2

GRIK1: Glutamate Ionotropic Receptor Kainate Type Subunit 1

GRIK4: Glutamate Ionotropic Receptor Kainate Type Subunit 4

GRIN3A: Glutamate ionotropic receptor NMDA type subunit 3A

GRM3: Glutamate Metabotropic Receptor 3

GWAS: Genome wide association studies

HERC2: Giant E3 ubiquitin protein ligase

ICD: International classification of diseases

IgG: Immunoglobulin G

IPA: Ingenuity pathway analysis

KEGG: Kyoto encyclopedia of genes and genomes

LH: Luteinizing hormone

LTP: Long-term potentiation

MAGI2: Membrane-Associated Guanylate Kinase Inverted 2

MAP1LC3B: Microtubule-associated proteins 1A/1B light chain 3B

MAP2K2: Mitogen-Activated Protein Kinase Kinase 2

MAPK1: Mitogen-Activated Protein Kinase 1

MAPKs: Mitogen-activated protein kinases

MDAG: Multi Disorder Autism Gene

MEK2: Mitogen-Activated Protein Kinase Kinase 2

MMR: Measles, mumps and rubella

NBC: Network-based candidates

NF1: Neurofibromin 1

NFKB: Nuclear factor κ-β

NR3C1: Nuclear Receptor Subfamily 3 Group C Member 1

NRG2: Pro-neuregulin-2

NRG3: Neuregulin 3

OXT: Oxytocin

PARK2: Parkin RBR E3 ubiquitin protein ligase

PBC: Process-based candidates

PBDEs: Polybrominated diphenyl ethers

PDD-NOS: Pervasive developmental disorder not otherwise specified

PDE4B: Phosphodiesterase 4B

PI3K: Phosphoinositide 3-kinase

PIK3R: Phosphatidylinositol 3-kinase regulatory subunit alpha

Pkcs: Protein kinases

PTEN: Phosphatase and tensin homologue

PTGS2: Prostaglandin-Endoperoxide Synthase 2

RNA: Ribonucleic acid

SCN1A: Sodium voltage-gated channel alpha subunit 1

SCN2A: Sodium voltage-gated channel alpha subunit 2

SETD2: SET Domain Containing 2, Histone Lysine Methyltransferase

SHANK2: SH3 and multiple ankyrin repeat domains 2

SHANK3: SH3 and multiple ankyrin repeat domains 3

SLC1A2: Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter)

SNP: Single-nucleotide polymorphism

SNRP: Small nuclear ribonucleoprotein polypeptide N

TCF4: Transcription Factor 4

TMS: Transcranial magnetic stimulation

ToM: Theory of mind

TP53: Tumor Protein P53

TRIM33: Tripartite Motif Containing 33

TRPV1: Transient Receptor Potential Cation Channel Subfamily V Member 1

TSC1: Tuberous sclerosis 1

TSC2: Tuberous Sclerosis 2

UBE3A: Ubiquitin protein ligase E3A

UK: United Kingdom

UPF2: Regulator of nonsense transcripts 2

US: United States

VEGF: Vascular endothelial growth factor

YWHAG: Tyr 3-Monooxygenase/Trp 5-Monooxygenase Activation Protein γ

3di: Developmental, dimensional and diagnostic interview

Chapter 2

Publications

El investigador sufre las decepciones, los largos meses pasados en una dirección equivocada, los fracasos. Pero los fracasos son también útiles, porque, bien analizados, pueden conducir al éxito. Y para el investigador no existe alegría comparable a la de un descubrimiento, por pequeño que sea ...

The researcher suffers the disappointments, the long months spent in the wrong direction, the failures. But failures are also useful, because, well analyzed, they can lead to success. And for the researcher, there is no joy comparable to that of a discovery, no matter how small ...

Sir Alexander Fleming

By virtue of Article 23, point 3, of the current Doctorate Regulations of the University of Jaén, the publications that constitute the core of this Doctoral Thesis are listed below.

These publications correspond to three scientific articles published in international journals indexed in the JCR (*Journal Citation Reports*), database produced by the ISI (*Institute for Scientific Information*).

2.1 A common molecular signature in ASD gene expression: following Root 66 to autism

- *Status:* Published
- *Title:* A common molecular signature in ASD gene expression: following Root 66 to autism
- *Authors:* Leticia Díaz Beltrán, Francisco José Esteban Ruiz and Dennis Paul Wall
- *Journal:* Translational Psychiatry
- *Publisher:* Nature Publishing Group
- *Volume:* 6. *Location:* e705. *Date:* Published online 5 January 2016
- *DOI:* `http://dx.doi.org/10.1038/tp.2015.112`
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- *Impact factor:* JCR 2015: 6.058; JCR 2016: 5.129; JCR 5-years average impact factor: 5.681
  - *Quartiles per knowledge areas:*
    - **Q1** in *Biological Psychiatry*
    - **Q1** in *Cellular and Molecular Neuroscience*
    - **Q1** in *Psychiatry and Mental Health*

OPEN

Citation: Transl Psychiatry (2016) **6**, e705; doi:10.1038/tp.2015.112

www.nature.com/tp

ORIGINAL ARTICLE

A common molecular signature in ASD gene expression: following Root 66 to autism

L Diaz-Beltran[1,2,3], FJ Esteban[3] and DP Wall[1,2,4]

Several gene expression experiments on autism spectrum disorders have been conducted using both blood and brain tissue. Individually, these studies have advanced our understanding of the molecular systems involved in the molecular pathology of autism and have formed the bases of ongoing work to build autism biomarkers. In this study, we conducted an integrated systems biology analysis of 9 independent gene expression experiments covering 657 autism, 9 mental retardation and developmental delay and 566 control samples to determine if a common signature exists and to test whether regulatory patterns in the brain relevant to autism can also be detected in blood. We constructed a matrix of differentially expressed genes from these experiments and used a Jaccard coefficient to create a gene-based phylogeny, validated by bootstrap. As expected, experiments and tissue types clustered together with high statistical confidence. However, we discovered a statistically significant subgrouping of 3 blood and 2 brain data sets from 3 different experiments rooted by a highly correlated regulatory pattern of 66 genes. This Root 66 appeared to be non-random and of potential etiologic relevance to autism, given their enriched roles in neurological processes key for normal brain growth and function, learning and memory, neurodegeneration, social behavior and cognition. Our results suggest that there is a detectable autism signature in the blood that may be a molecular echo of autism-related dysregulation in the brain.

Translational Psychiatry (2016) **6,** e705; doi:10.1038/tp.2015.112; published online 5 January 2016

INTRODUCTION

Autism is regarded as one condition among a genetically heterogeneous group of neurodevelopmental syndromes with high prevalence[1] that has a wide range of phenotypes, collectively grouped together as autism spectrum disorder (ASD). The unifying clinical features across the spectrum involve fundamental impairments in social interaction, communication deficits and highly restrictive interest and/or repetitive behaviors.[2,3] Although there is no unifying hypothesis about the molecular pathology of autism, it is clear that the disorder is highly heritable and results from the combination of genetic, neurologic, immunologic and environmental factors. However, it remains unclear whether its genetic component stems from the combination of a few common variants or of many rare variants.[4,5]

Recent advances in genetics, genomics, developmental neurobiology and systems biology have offered important insights into the molecular agents and biological mechanisms responsible for ASD. Microarray technologies and next-generation sequencing have enabled high-throughput discovery of genes likely to be involved in the molecular pathology of autism.[5–8] However, as the success in discovery has risen, the number of candidate genes with associated risk for ASD has also stretched well into the hundreds.[9,10] As of December 2014, 667 genes have been implicated in autism (https://gene.sfari.org/autdb/HG_Home.do). Despite the large amounts of data now available, the general lack of replication across studies suggests that more data will be needed to fully characterize the genetic models responsible for the various forms of autism.

These high-throughput and large-scale efforts have confirmed that autism is a multisystem and heterogeneous condition. Thus, understanding the complex genetic architecture of ASD must involve, among other things, the study of autism gene expression across different tissues using integrative approaches. The majority of gene expression experiments conducted so far have been on blood-derived cells and to a lesser extent postmortem brain tissue from autism cases and matched controls. More recent approaches have examined regulatory patterns in induced pluripotent stem cells forming neurons from individuals with autism. Individually, these studies have advanced our understanding of molecular systems involved in either the cause or effect of autism. We propose and test here the notion that together these experiments may help refine our understanding of genes and pathways important in onset and maintenance of autism. Specifically, we perform an integrated systems biology analysis of all published autism gene expression studies to test whether a common signature representative of ASD exists and ultimately if it can be detected in both blood and brain.

MATERIALS AND METHODS

Experiments and gene lists

To compile a complete set of published and publically available gene expression experiments we used Nextbio,[11] an ontology-based platform that provides global collections of high-throughput public data that meet four criteria: broad coverage of genes, existence of a control group, access to raw or normalized data and supply of sample annotations. We downloaded gene expression data and derived lists of differentially expressed genes from 27 case–control biosets of 9 independent

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E-mail: dpwall@stanford.edu
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2.2 Comorbid Analysis of Genes Associated with Autism Spectrum Disorders Reveals Differential Evolutionary Constraints

- *Status:* Published
- *Title:* Comorbid Analysis of Genes Associated with Autism Spectrum Disorders Reveals Differential Evolutionary Constraints
- *Authors:* Maude M. David, David Enard, Alp Ozturk, Jena Daniels, Jae-Yoon Jung, Leticia Díaz Beltrán and Dennis Paul Wall
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PLOS ONE

RESEARCH ARTICLE

Comorbid Analysis of Genes Associated with Autism Spectrum Disorders Reveals Differential Evolutionary Constraints

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

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Abstract

The burden of comorbidity in Autism Spectrum Disorder (ASD) is substantial. The symptoms of autism overlap with many other human conditions, reflecting common molecular pathologies suggesting that cross-disorder analysis will help prioritize autism gene candidates. Genes in the intersection between autism and related conditions may represent non-specific indicators of dysregulation while genes unique to autism may play a more causal role. Thorough literature review allowed us to extract 125 ICD-9 codes comorbid to ASD that we mapped to 30 specific human disorders. In the present work, we performed an automated extraction of genes associated with ASD and its comorbid disorders, and found 1031 genes involved in ASD, among which 262 are involved in ASD only, with the remaining 779 involved in ASD and at least one comorbid disorder. A pathway analysis revealed 13 pathways not involved in any other comorbid disorders and therefore unique to ASD, all associated with basal cellular functions. These pathways differ from the pathways associated with both ASD and its comorbid conditions, with the latter being more specific to neural function. To determine whether the sequence of these genes have been subjected to differential evolutionary constraints, we studied long term constraints by looking into Genomic Evolutionary Rate Profiling, and showed that genes involved in several comorbid disorders seem to have undergone more purifying selection than the genes involved in ASD only. This result was corroborated by a higher dN/dS ratio for genes unique to ASD as compare to those that are shared between ASD and its comorbid disorders. Short-term evolutionary constraints showed the same trend as the pN/pS ratio indicates that genes unique to ASD were under significantly less evolutionary constraint than the genes associated with all other disorders.

Introduction

Autism Spectrum Disorder (ASD) is a heritable developmental disorder that affects one in sixty-eight children [1]. Its prevalence is rising at an alarming rate, up from one in eighty-eight

2.3 Cross-disorder comparative analysis of comorbid conditions reveals novel autism candidate genes

- *Status:* Published
- *Title:* Cross-disorder comparative analysis of comorbid conditions reveals novel autism candidate genes
- *Authors:* Leticia Díaz Beltrán, Francisco José Esteban Ruiz, Maya Varma, Alp Ortuzk, Maude David and Dennis Paul Wall
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Diaz-Beltran et al. BMC Genomics (2017) 18:315
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BMC Genomics

RESEARCH ARTICLE Open Access

Cross-disorder comparative analysis of comorbid conditions reveals novel autism candidate genes

Leticia Diaz-Beltran[1,2,3†], Francisco J. Esteban[3†], Maya Varma[1,2], Alp Ortuzk[1,2], Maude David[1,2] and Dennis P. Wall[1,2,4*]

Abstract

Background: Numerous studies have highlighted the elevated degree of comorbidity associated with autism spectrum disorder (ASD). These comorbid conditions may add further impairments to individuals with autism and are substantially more prevalent compared to neurotypical populations. These high rates of comorbidity are not surprising taking into account the overlap of symptoms that ASD shares with other pathologies. From a research perspective, this suggests common molecular mechanisms involved in these conditions. Therefore, identifying crucial genes in the overlap between ASD and these comorbid disorders may help unravel the common biological processes involved and, ultimately, shed some light in the understanding of autism etiology.

Results: In this work, we used a two-fold systems biology approach specially focused on biological processes and gene networks to conduct a comparative analysis of autism with 31 frequently comorbid disorders in order to define a multi-disorder subcomponent of ASD and predict new genes of potential relevance to ASD etiology. We validated our predictions by determining the significance of our candidate genes in high throughput transcriptome expression profiling studies. Using prior knowledge of disease-related biological processes and the interaction networks of the disorders related to autism, we identified a set of 19 genes not previously linked to ASD that were significantly differentially regulated in individuals with autism. In addition, these genes were of potential etiologic relevance to autism, given their enriched roles in neurological processes crucial for optimal brain development and function, learning and memory, cognition and social behavior.

Conclusions: Taken together, our approach represents a novel perspective of autism from the point of view of related comorbid disorders and proposes a model by which prior knowledge of interaction networks may enlighten and focus the genome-wide search for autism candidate genes to better define the genetic heterogeneity of ASD.

Keywords: Autism Spectrum Disorder, Autism sibling disorders, Gene set enrichment, Process enrichment, Comparative network analysis, Systems biology

Background

Autism spectrum disorder (ASD) encompasses a group of complex neurodevelopmental disorders characterized, in different ranges, by impaired social interaction, difficulties in verbal and non-verbal communication and restricted, stereotyped and repetitive behaviors. Its symptoms begin in early childhood and persist through adulthood, affecting daily functioning [1].

This lifelong condition, 4 times more common in males than females, is one of the fastest-growing developmental disorders worldwide and its prevalence continues to increase at an alarming rate. In fact, large-scale surveys estimated median rates of increase at 1–2% [1–8]. The US Center for Disease Control and Prevention (CDC) [9] now indicates that 1 in 68 American children have ASD. In addition, the 2014 National Health Interview Survey,

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Chapter 3

Supplementary material

Por encima de todo, mi vida es la investigación.

Above all, my life is research.

Margarita Salas

3.1 Appendix 1. Supplementary material

Here below are enclosed the links of the supplementary figures and tables of the three publications that constitute the core of this Ph.D. Thesis.

3.1.1 A common molecular signature in ASD gene expression: following Root 66 to autism

Supplementary Information accompanies the paper on the Translational Psychiatry website `https://www.nature.com/articles/tp2015112#MOESM246`

- Supplementary Information (DOC 251 kb)
- Supplementary Figure 1 (JPG 1187 kb)
- Supplementary Figure 2 (JPG 227 kb)

- Supplementary Figure 3 (JPG 356 kb)
- Supplementary Figure 4 (JPG 584 kb)
- Supplementary Figure 5 (JPG 350 kb)
- Supplementary Figure 6 (JPG 103 kb)

3.1.2 Comorbid Analysis of Genes Associated with Autism Spectrum Disorders Reveals Differential Evolutionary Constraints

Supporting information (as provided by the publisher)

- S1 Fig. PDF Standard error of each p-value calculated in Fig 1 multi-scale bootstrap.
- S2 Fig. PDF Fitting curve for the multiscale bootstrap performed Fig 1 for each cluster.
- S1 Table XLSX Correspondence table for ICD-9 codes, ICD-9 disorder, Phenopedia terms and MeSH terms.
- S2 Table XLSX Correspondence between KEGG Orthologs, hsa (KEGG) and Symbol ID.
- S3 Table XLSX Complete list of pathways described in Fig 3.
- S4 Table XLSX pN value, pS value, and dN/dS ratio used in this study.
- S5 Table XLSXComplete list of genes involved in each disorder analyzed.

3.1.3 Cross-disorder comparative analysis of comorbid conditions reveals novel autism candidate genes

Additional files (as provided by the publisher)

- Additional file 1: Table S1. Information summary of the datasets selected. (DOCX 12 kb)

- Additional file 2: Figure S1. Box plots showing the distribution of the samples of each dataset after preprocessing; median-centered values indicate that the data are normalized and cross-comparable. (PDF 5181 kb)

- Additional file 3: Table S2. Complete list of comorbid conditions to autism. Autism sibling disorders are highlighted in blue. (XLSX 16 kb)

- Additional file 4: Table S3. Total number of genes of each comorbid condition utilized for this study. Highlighted in blue are ASD sibling disorders. (XLSX 135 kb)

- Additional file 5: Table S4. Comorbid disorders integrating each group generated by the bootstrap analysis (Additional file 6: Figure S2), along with their Mean Jaccard Coefficient value. The different groups of disorders generated by our bootstrap procedure corresponds to the disorder clusters obtained in our original gene-based dendrogram (Fig. 1). Groups 1, 2 and 3 have the highest Mean Jaccard values meaning they are the most robust and stable groupings of the tree. Group 2 coincides with the cluster conformed by the autism sibling disorders with a highly significant Mean Jaccard value of approximately 0.785. (DOCX 13 kb)

- Additional file 6: Figure S2. First two Multidimensional Scaling (MDS) dimensions of our dataset generated by MDS on a dissimilarity matrix using Jaccard Coefficient with k equal to 6. Each group is highlighted in a different color and the disorders conforming them are detailed in Additional file 5. Table S4, along with their corresponding mean Jaccard Coefficient value. The autism sibling comorbid disorders are clustered together in group 2 (PDF 180 kb)

- Additional file 7: Table S5. STRING edge summary for each member of the ASD sibling group. (XLSX 8411 kb)

- Additional file 8: Table S6. The multi disorder autism gene set (MDAG) and the sibling comorbid conditions where these genes are found. (XLSX 25 kb)

- Additional file 9: Figure S3. A. The complete network of autism candidate genes. The MDAG genes are highlighted in yellow and their interactions in red; these are the genes that occur in one or more of the autism sibling comorbid disorders, circumscribed in Fig. 1. B. The highly interconnected subcomponent conformed by the MDAG genes, separated from the autism network. (TIF 406700 kb)

- Additional file 10: Table S7. Biological processes for which the Multi-disorder component of the autism gene set (MDAG) were enriched. Identities of the MDAG genes overrepresented in the processes as well as the corrected p-values for the enrichment scores are provided. Enrichment was calculated using the biological processes only found among the MDAG genes and not found among the sibling disorders. (XLSX 50 kb)

- Additional file 11: Table S8. Identities of the differentially expressed PBC, along with their corresponding q-values, the biological processes where they are involved and the comorbid disorders where they are implicated. Also, PBC significantly differentially expressed in each dataset and in all the three datasets. (XLSX 210 kb)

- Additional file 12: Figure S4. P-value and q-value histograms and q-plots from the multiple test correction analyses performed on the PBC and NBC to verify whether they were significantly differentially regulated in autistics in comparison to controls. (PDF 11411 kb)

- Additional file 13: Table S9. Complete list of NBC, along with their q-values, MDAG interactors and comorbid disorders where they are present. Also, NBC significantly differentially expressed in each dataset and in all the three datasets. (XLSX 185 kb)

- Additional file 14: Table S10. Complete list of genes present in the intersection of PBC intersection NBC, along with the biological processes where they are involved, MDAG

interactors and comorbid disorders where they are present. Also, PBC intersection NBC significantly differentially expressed in each dataset and in all the three datasets. (XLSX 226 kb)

3.2 Appendix 2. Other publications

In this section are included other research works related to systems biology and neurological conditions that were published during the development of this Doctoral Thesis.

3.2.1 Systems Biology as a comparative approach to understand complex gene expression in neurological diseases

- *Status:* Published
- *Title:* Systems Biology as a comparative approach to understand complex gene expression in neurological diseases
- *Authors:* Leticia Díaz Beltrán, Carlos Cano, Francisco José Esteban Ruiz and Dennis Paul Wall
- *Journal:* Behavioral Sciences
- *Publisher:* MDPI Journals
- *Volume:* 3. *Location:* 253-272. *Date:* Published online 2013 May 21
- *DOI:* `http://dx.doi.org/10.3390/bs3020253`
- *ISSN:* 2076-328X
- *Abstracted/indexed in:* PubMed, MEDLINE, PubMed Central, Scopus, ISI Web of Science, DOAJ (Directory of Open Access Journals), and British Library, among others.

Behav. Sci. **2013**, *3*, 253–272; doi:10.3390/bs3020253

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Review

Systems Biology as a Comparative Approach to Understand Complex Gene Expression in Neurological Diseases

Leticia Diaz-Beltran [1,3], Carlos Cano [2], Dennis P. Wall [3] and Francisco J. Esteban [1,3,*]

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Received: 9 April 2013; in revised form: 8 May 2013 / Accepted: 16 May 2013 / Published: 21 May 2013

Abstract: Systems biology interdisciplinary approaches have become an essential analytical tool that may yield novel and powerful insights about the nature of human health and disease. Complex disorders are known to be caused by the combination of genetic, environmental, immunological or neurological factors. Thus, to understand such disorders, it becomes necessary to address the study of this complexity from a novel perspective. Here, we present a review of integrative approaches that help to understand the underlying biological processes involved in the etiopathogenesis of neurological diseases, for example, those related to autism and autism spectrum disorders (ASD) endophenotypes. Furthermore, we highlight the role of systems biology in the discovery of new biomarkers or therapeutic targets in complex disorders, a key step in the development of personalized medicine, and we demonstrate the role of systems approaches in the design of classifiers that can shorten the time for behavioral diagnosis of autism.

Keywords: systems biology; neurological diseases; gene expression; autism; autism spectrum disorders; network analysis; protein-protein interactions; translational bioinformatics; behavioral diagnosis

3.2.2 Fractal analysis in neurological diseases

- *Status:* Published
- *Title:* Fractal analysis in neurological diseases
- *Authors:* Francisco José Esteban Ruiz, Leticia Díaz Beltrán and Antonio Di Ieva
- *Book:* The Fractal Geometry of the Brain
- *Editor:* Antonio Di Ieva
- *Publisher:* Springer, Springer Nature
- *Year:* 2016
- *Pages:* 1199-2122
- *DOI:* `http://dx.doi.org/10.1007/978-1-4939-3995-4_13`
- *ISBN:* 9781493939954

Springer Series in Computational Neuroscience

Antonio Di Ieva *Editor*

The Fractal Geometry of the Brain

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Chapter 13
Fractal Analysis in Neurological Diseases

Francisco J. Esteban, Leticia Díaz-Beltrán, and Antonio Di Ieva

Abstract Over the last decades, fractal analysis has been applied to the study of the spatial and temporal complexity of a wide range of objects in biology and medicine, including the irregular and complex patterns of the nervous system. In clinical neurosciences, fractal geometry has emerged as a powerful tool to objectively analyze and quantify the intricate structures comprising the topological and functional complexity of the human brain, shedding light on the understanding of the brain function at a systems level. The fractal approach has the potential to allow physicians and scientists to predict clinical outcomes, classification between normal and pathological states, and, ultimately, the identification and diagnosis of certain neurological conditions. In this chapter, the main applications of fractal analysis into clinical neurosciences are reviewed, with special emphasis on the diagnostic precision of the fractal dimension value in different neurological diseases.

Keywords Brain • Clinical neurosciences • Fractal dimension • Fractal analysis • Magnetic resonance imaging • Neurology

In neurosciences, fractal analysis is used to measure the scaling inherent to neurological systems (from anatomic to histological structures), providing an index, the fractal dimension (FD), to estimate the topological complexity of the given object. Different types of neural structures, from neurons to complex networks, can be characterized as structural or dynamical fractals to quantify the intrinsic complexity. In this perspective, the spatial properties of the components of the nervous system, both at the macroscopic and microscopic levels, can be viewed as geometric

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Contenido

Integración y análisis de datos mediante biología de sistemas

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Resumen

En los últimos años se ha producido un desarrollo significativo de las técnicas empleadas en biología molecular y celular, especialmente en el campo de las llamadas tecnologías *ómicas* de alta resolución. Dicho avance supone un reto para la investigación biomédica y biotecnológica pues, a pesar de sus múltiples ventajas, genera un gran volumen de información que debe ser analizado cuidadosamente utilizando potentes herramientas computacionales. En este sentido, la biología de sistemas, encargada de la integración y análisis de datos a distintos niveles, se está convirtiendo en un referente a la hora de estudiar la complejidad de los sistemas biológicos en diferentes situaciones experimentales o patológicas. Este nuevo enfoque de carácter multidisciplinar hace posible, entre otras diversas aplicaciones, la detección de biomarcadores y predictores de enfermedad.

Se presenta una descripción de las herramientas actuales para el análisis de datos procedentes de tecnologías *ómicas* (R y paquetes de Bioconductor; TM4 *software suite*; STRING; Cytoscape) acompañada de ejemplos de aplicación con datos procedentes de bases de datos públicas (principalmente *Gene Expression Omnibus*), con el objetivo de poner de manifiesto su importancia.

Palabras clave: biología computacional, biomedicina, expresión génica, *ómicas,* redes moleculares

Abstract

Molecular and cellular biology techniques have been suffering a significant development in the last years, especially in the field of the so-called "omics" technologies. This advance represents a challenge for biomedical and biotechnological research, mainly due to the many advantages it generates when a large amount of information needs to be analyzed using powerful computational tools. Systems biology, that allows the integration and interpretation of omics data at different levels, is becoming crucial for studying the complexity underlying biological systems in different experimental and pathological situations. This new multidisciplinary approach has led to the detection of biomarkers and disease predictors, among other several applications.

In this chapter, the current tools for omics data analysis (mainly R and Bioconductor packages; TM4 software suite; STRING; Cytoscape) are presented including examples of applications using public databases datasets (such as Gene Expression Omnibus) and with the final aim of highlighting their relevance.

Keywords: *computational biology, biomedicine, gene expression, omics, molecular networks*

Introducción

Biología de sistemas, un enfoque interdisciplinar

El desarrollo tecnológico y metodológico que ha tenido lugar en las últimas décadas ha supuesto un espectacular avance en el campo de la biología molecular y celular. Dicho avance se debe en parte a la aparición de las tecnologías *ómicas* de alta resolución, que analizan entidades (que pueden ser conjuntos de genes, proteínas u organismos, entre otros) de manera global y de forma que hablamos de genómica, proteómica, metabolómica o epigenómica. Así pues, se plantea un enfoque holístico que complementa a los análisis tradicionales reduccionistas, en los que se consideraba un único elemento como objeto de es-

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