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IN

BIOLOGICAL SCIENCES

SCHOOL OF SCIENCE, ENGINEERING AND ENVIRONMENT

INVESTIGATION OF DE-NOVO COPY NUMBER VARIANTS IN PATIENTS WITH AUTISM SPECTRUM DISORDER IN VIETNAM

By

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DECLARATION

I, Hoa Thi Phuong Bui, declare that the thesis entitled "Investigating *de novo* copy number variants in patients with autism spectrum disorder in Vietnam" and the work presented in the research carried out at Vinmec healthcare system and University of Salford under the guidance of Dr Arijit Mukhopadhyay, Dr Ian Goodhead and Prof. Liem Thanh Nguyen are my own. No part of this work has been previously submitted at any institution.

I further declare that the materials obtained from other sources have been acknowledged in the thesis.

Hanoi, 18th October, 2021

PhD candidate

Hoa Thi Phuong Bui

Abbreviations

ABA	Applied Behavior Analysis
ABC	Aberrant Behavior Checklists
aCGH	Microarray-based comparative genomic hybridization
ACMG	American College of Medical Genetics and Genomics
ADHD	Anxiety/attention deficit hyperactivity disorder
ADM	Aberration detection method
ADOS	Autism diagnostic observation schedule
AE	Acetate/Ethylenediaminetetraacetic acid
AS	Asperser's syndrome
ASD	Autism spectrum disorder
ASQ	Autism screening questionnaire
BMMNC	Bone marrow mononuclear cell
BP	Biological process
CARS	Childhood autism rating scale
CC	Cellular component
CDC	Centers for Disease Control and Prevention
CGI	Clinical global impression
CMA	Chromosome array
CNV	Copy number variation
CP	Cerebral palsy
DD	Developmental delay

DECIPHER	Database of chromosomal imbalance and phenotype in Humans using ensemble resources
DGV	Database of genomic variants
DNA	Deoxyribonucleic acid
DSM-V	Diagnostic and statistical manual of mental disorders
EDTA	Ethylenediaminetetraacetic acid
ESDM	Early Start Denver Model
G-CSF	Granulocyte-colony stimulating factor
gDNA	Genomic deoxyribonucleic acid
GI	Gastrointestinal
GO	Gene ontology
ID	Intellectual disability
IDEA	Individuals with disabilities education act
IQ	Intellectual functioning
ISCA	International standards for cytogenomic arrays
IVF	In vitro fertilization
EP	Epilepsy
FDG	2-fluoro-2-deoxy-D-glucose
FISH	Fluorescence in situ hybridization
FMN	Fragile mental retardation
FSC	Fetal stem cell
hASC	Human adipose derived stem cell
hNPC	Human neural progenitor cell
HSC	Hematopoietic stem cell

HUCB	Human umbilical cord blood
ICD	International statistical classification of diseases and related health problems
IL	Interleukin
ISAA	Indian Scale for Assessment of Autism
M-CHART	Modified checklist for autism in toddlers-revised
MECP2	Methyl-CpG binding protein 2
MF	Molecular function
MLPA	Multiple ligation-dependent probe amplification
MNC	Mononuclear cell
MR	Mental retardation
MRI	Magnetic resonance Image
MSC	Mesenchymal stem cell
NK	Natural killer
OMIM	Online Mendelian inheritance in Man
PCR	Polymerase chain reaction
PDD-NOS	Pervasive developmental disorder-not otherwise specified
PEM	Pair-end mapping
PET-CT	Positron emission tomography – computed tomography
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
QC	Quanlity control
RD	Read depth
RNA	Acid ribonucleic

SD	Standard deviation
SFARI	Simons Foundation Autism Research Initiative
SHANK	SH3 and multiple ankyrin repeat domain
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
SPECT	Single-photon emission computed tomography
SR	Split read
SUV	Standardized uptake values
TBE	Tris/Borate/Ethylenediaminetetraacetic acid
TSC	Tuberous sclerosis complex
UC-MSC	Umbilical cord mesenchymal stem cell
UCSC	University of California Santa Cruz
UK	United Kingdom
USA	United States of America
VEFG	Vascular endothelial growth factors
VPA	Valproic acid
VUS	Variant of uncertain significant
WES	Whole exome sequencing
WGS	Whole genome sequencing

INVESTIGATION OF DE-NOVO COPY NUMBER VARIATIONS IN PATIENTS WITH AUTISM SPECTRUM DISORDER IN VIETNAM

Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with a prevalence of approximately 1% children worldwide. ASD is characterized by deficits in social communication and interaction and the presence of restricted interests and repetitive behaviours. Genetic alterations contributing to increasing risk of ASD have been reported. Early genetic screening, especially for families with a positive ASD history, could aid the early diagnosis and potentially more effective disease management strategies. Copy number variants (CNVs) are the alterations in the structure of chromosome and reported as a significant contribution to the pathogenesis of ASD. Currently, genome-wide DNA microarray (e.g. microarray comparative genomic hybridization (aCGH)) is considered as the first-tier screen for genetic aberrations in autistic children, albeit with a limited success (around 10% in studies primarily based on patients of Caucasian origin). Currently, there is no comprehensive study on the diagnostic potential of aCGH in autistic children from Vietnam. This study aims to investigate the possible role of CNV in Vietnamese patients with a clinical diagnosis of ASD. One hundred trios (both parents and at least one child) were recruited where in each trio the child was clinically diagnosed with ASD while the parents were not clinically affected. *MECP2* and *FMR1* DNA tests were performed for excluding Rett Syndrome and Fragile X syndrome as possible causes, respectively. The aCGH test was performed on all patients as well as their parents to identify de-novo CNVs in the patients. We detected 442 non-redundant CNVs in 100 patients with 210 (47.5%) identified as *de novo* in origin. We identified five variants of uncertain significance (VUS) as well as pathogenic *de novo* CNVs (two duplication and three deletion CNVs) in seven patients (four males and three females) related to autism based on the SFARI (Simons Foundation Autism Research Initiative) database. In six patients, four

known pathogenic CNVs were identified (diagnostic success 6%). We found the highest (3%) contribution of deletions involving *SHANK3* gene, which could pave the way for future diagnostics focused on this gene alone. These findings provide initial information for building an aCGH screening test for Vietnamese autistic children and identifying the relationship between genetic alterations and the effectiveness of stem cell transplantation – bringing a new strategy for ASD management in Vietnam.
Key words: Autism Spectrum Disorder, Microarray CGH, Vietnam,

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CHAPTER 1: INTRODUCTION

1.1. Human brain: Structure and functions

The brain is one of the most complex organs in our body composed by billions of neurons (Jawabri KH, 2021). It controls functions of the whole body including thoughts, memory and speech, movement, functions of many organs in our body by connecting with the outside through five senses: sight, smell, touch, taste and hearing – often many at one time. These information are collected, interpreted and stored through particular areas of the brain that helps the body responses to the outside world and embodying the essence of the mind and soul. It governs many things including abstract features such as intelligence, creativity, emotion and memory (S, 1992). The brain is composed of three main parts: the cerebrum, cerebellum and brainstem, each with multiple sub-parts that are protected by the skull (Jawabri KH, 2021). Cerebrum is the largest part of the brain and divided into two hemispheres: the right and the left. They are connected by the corpus callosum that transmits messages from one side of the brain to the other side of the body. But each hemisphere has its own functions. For example, the left controls speech and comprehension while the right controls creativity and artistic attributes (Madhukar N, 2021). Figure 1 shows the overview of human brain structure.

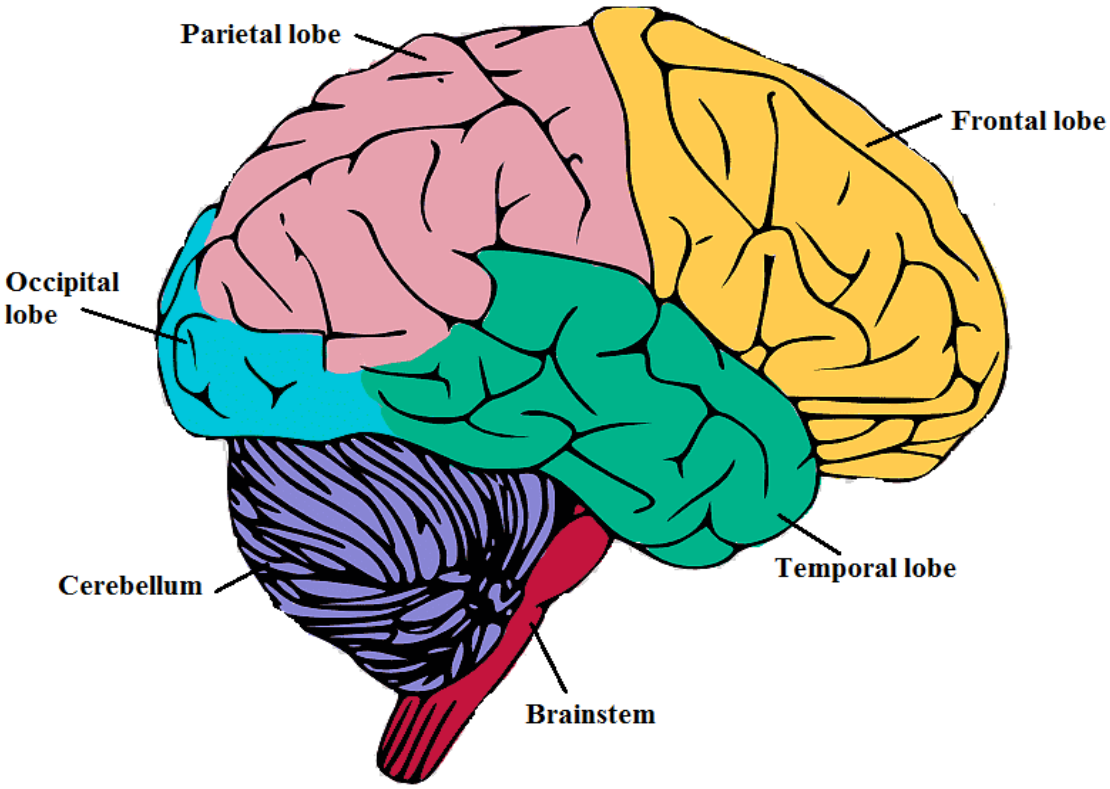

Figure 1: Structure of human brain (modified from (Klipartz, 2020))

1.1.1. The cerebrum

Also called the cerebral cortex, is the largest part of the human brain. It is the conditioned reflex centre, which controls higher brain functions such as action and thought. The cerebrum has two parts, grey surface made up by nerve cells called cortex with white matter (white nerve fibres) beneath. The cortex contains ~16 billion neurons arranged in specific layers. The folding of the cortex helps to improve the area of the brain's surface that in turn allows fitting in more neurons and hence enabling better and increasingly complex functions. Folds and grooves form specific brain regions. Each fold is called a gyrus and between each groove is called a sulcus. The cerebrum has four sections: the frontal lobe, the parietal lobe, the occipital lobe and the temporal lobe.

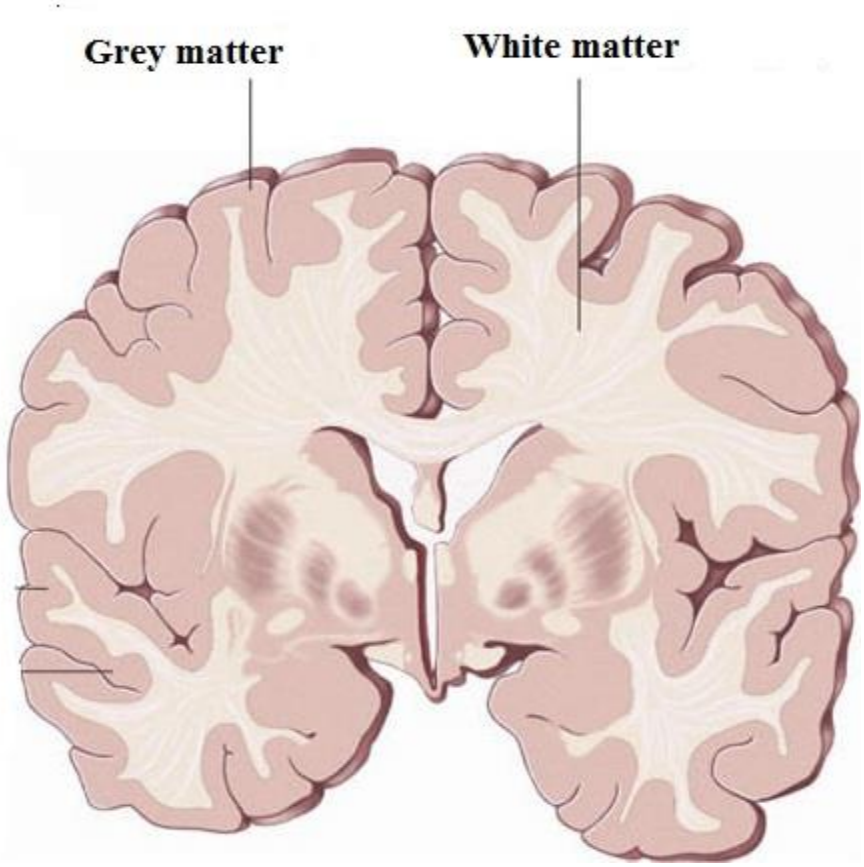

Figure 2: Structure of cortex with grey surface and white nerves beneath (modified from (Hitziger, 2015))

1.1.1.1. Frontal lobe

Frontal lobe is the largest lobe, located in front of the cerebral hemispheres. It has particular functions for our body including:

Prospective memory: a type of memory relates to plan making from basic daily plan to lifelong plans (Neulinger et al., 2016).

Speech and language: Broca's area which relates to speech production is in the posterior inferior frontal gyrus. A recent research reported that sensory representations from the temporal cortex go through the Broca's area and come to the motor cortex (Flinker et al., 2015).

Personality: Several studies reported the five sub-types of personality changes after frontal lobe injuries including executive disturbances, disturbed social behaviour, emotional dysregulation, hypo-emotionality/de-energization, and distress (Jawabri KH, 2021, Barrash et al., 2018)

Decision making: the ability for decisions that involves reasoning, learning and creativity. A study proposed PROBE model to understand how the decision-making process occurs in the frontal lobes (Collins and Koechlin, 2012).

Movement control: motor cortex in the frontal lobe is divided into two regions including primary and non-primary motor areas but the exact function and role to the movement of each regions is still not well-studied (Chouinard and Paus, 2006).

1.1.1.2. Parietal lobe

Parietal lobe is located posterior to the frontal lobe and superior to the temporal lobe. It is classified into two subtype regions including the anterior and posterior lobes.

The primary sensory cortex located in the anterior parietal lobe receives the majority of the sensory inputs from the thalamus and interprets the simple somatosensory signals, such as touch, position, vibration, pressure, pain and temperature (Jawabri KH, 2021).

The posterior parietal lobe contains the superior and inferior regions. The somatosensory association cortex in the superior region involves in higher-order functions like motor planning activities while the secondary somatosensory cortex in the inferior region receives the somatosensory inputs from the thalamus and integrates these information combined with other major modalities such as visual inputs, auditory inputs to form higher-order complex functions including sensorimotor planning, learning, language, spatial recognition and stereognosis (Jawabri KH, 2021).

1.1.1.3. Temporal lobe

The temporal lobe lied posterior to the frontal lobe and inferior to the parietal lobe has two surfaces: the lateral and medial surfaces (Jawabri KH, 2021).

The lateral surface: The Heschl gyrus located in the lateral surface of superior temporal plane contains the primary auditory cortex that is responsible for translating and processing all sounds and tones. The middle temporal gyrus has primarily involved in sound recognition, semantic retrieval, semantic memory, semantic control network, classical sensory language, decoding gaze directions and speech. The inferior temporal gyrus relates to visual perception and facial perception by containing the ventral visual pathway.

The medial surface: contains many important structures such as Hippocampus, Entorhinal, Perirhinal, Parahippocampal cortex which relate to declarative memory (long-term memory including remembering the concepts, ideas, events).

1.1.1.4. Occipital lobe

The occipital lobe which is the smallest lobe is located in the posterior region of the parietal and temporal lobes. It is responsible for visual processing and interpretation. The visual cortex receives the information from the thalamus, processes, interprets and sent to the other regions of the brain for further analysis. It helps us to determine, recognize and compare the objects to each other (Jawabri KH, 2021).

1.1.1.5. Nerve cells

The human brain contains about 10 billions neurons (nerve cells) and each neuron can have a thousand connections with others. These cells sense features from both external and internal environment and transmit information to the brain for processing and storage (Lodish H, 2000). The structure and function of these cells are well understood. Neurons have many sizes and shapes but all consist of a cell body, dendrites and an axon (figure 3). Information is conveyed by both electrical and chemical signals. Electric signal process information within a cell while chemical signals transfer information from cell to

cell. There are several nerve cells including sensory neurons, interneurons, and motor neurons responsible for different functions. Sensory neurons collect and process information from the environment such as light, touch, and sound into electric signals. These electric signals are then transferred to chemical signals, and passed to interneurons. Within these cells, chemical signals are again converted to electric signals and transmitted to motor neurons or other types of cells (Lodish H, 2000). Neurons transmit their energy (signal) to each other across a gap-junction called a synapse (figure 4). Three components of a synapse combination including presynaptic endings (neurotransmitters), synaptic clefts (gap), and postsynaptic endings (receptors) (Guy-Evans, 2021). These signals are passed to the cell body and selective information will be passed to the end of an axon. The transfer of information by releasing of chemical substances. These chemicals are call neurotransmitters. Neurotransmitters contained in sacs are crossed into the synapse and fit into the special receptors on the receiving nerve cell for passing the message. Each neurotransmitter can only bind to a specific matching receptor as a key and a lock. Postsynaptic neurons can also communicate back to the presynaptic neurons for sending a feedback that they need to change how often or how much neurotransmitter is released. This means synapses are able to communicate bi-directionally. Synapses can be either chemical or electrical and are essential for neural activity functions. They play a vital role in learning and memory formations (Guy-Evans, 2021).

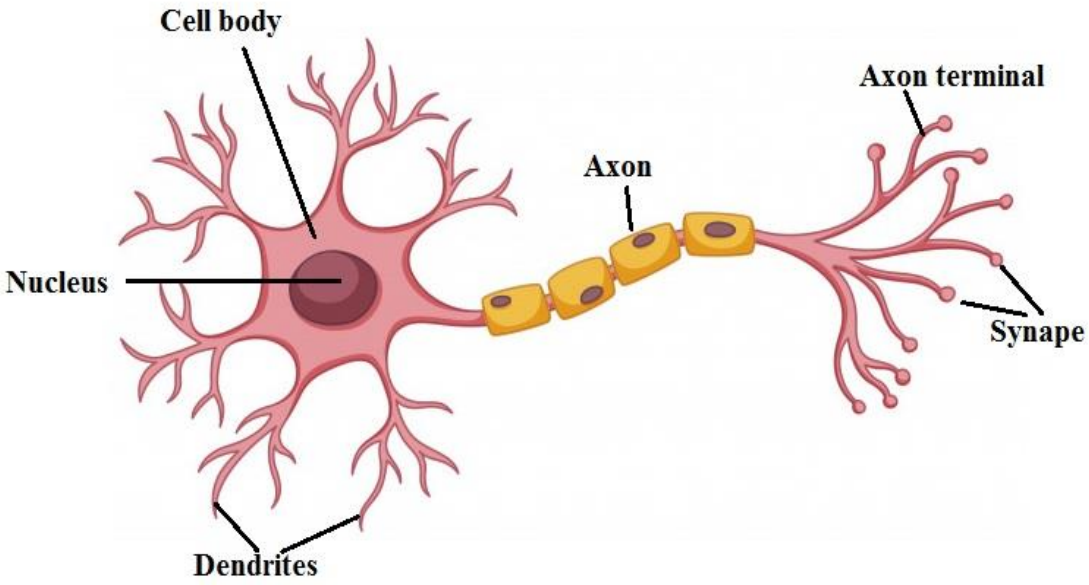

Figure 3 Structure of nerve cell (modified from (Freepik, 2020))

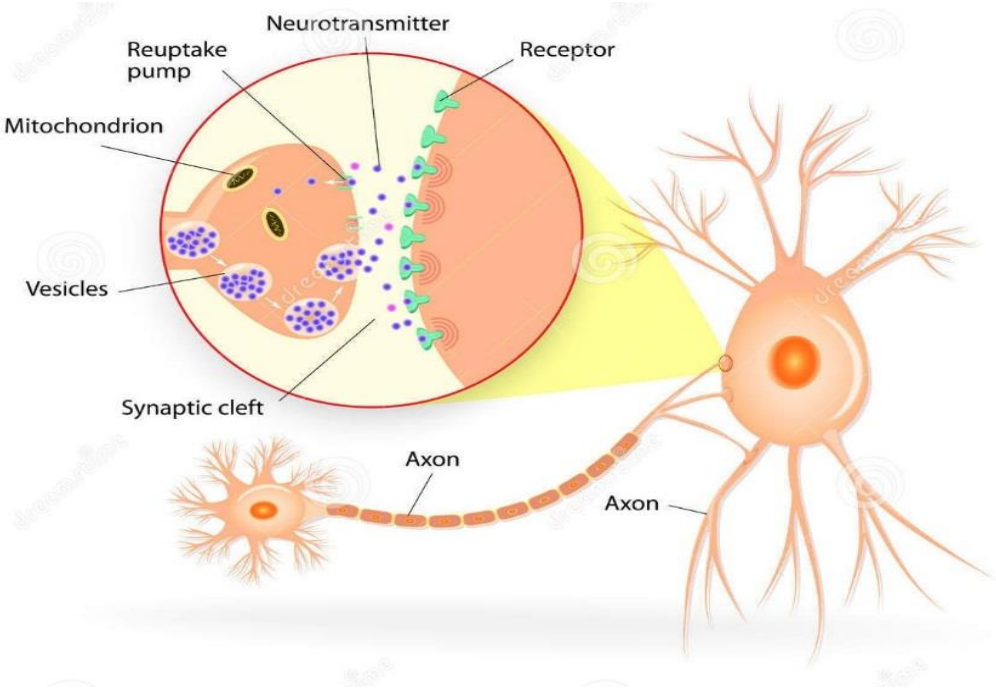

Figure 4: Synaptic transmission (modified from (Dreamstime, 2020))

1.1.2. Brainstem

The brainstem including midbrain, the pons and the medulla is composed of several nerves, pathways, reflex centres and nuclei. The midbrain is the smallest part of the brain and located most centrally within the cranial cavity. It is in the brainstem between the pons caudally and the diencephalon (thalamus, hypothalamus, epithalamus and subthalamus). The midbrain mediates the reflexes of miosis and mydriasis and play an essential role in sensory and motor control pathways. It is also responsible for the connection of nervous system between the spinal cord and brain (Caminero F, 2020).

1.1.3. Cerebellum

The Cerebellum which is the largest part of the hindbrain is located in the posterior cranial fossa, behind the fourth ventricle, the pons, and the medulla oblongata. It has two hemispheres connected by the vermis. The cerebellar cortex is made up by many folds that is composed by gray matter cover and an inner white matter (Jimsheleishvili S, 2021). It is associated with regulation and coordination of movement, posture and balance as well as the cardiac, respiratory and vasomotor centres.

1.1.4. Alterations of brain structure and functions in autism

Alterations in brain structure and functions may contribute to the development of autism, for example: (1) abnormalities in the inferior frontal gyrus (Broca's area), superior temporal sulcus, and Wernicke's area might be related to defects in social language processing and social attention (Redcay, 2008); (2) the frontal lobe, superior temporal cortex, parietal cortex, and amygdala might mediate impairments of social behaviours (Adolphs, 2001, Kim et al., 2010); and (3) the orbitofrontal cortex and caudate nucleus have been associated with restricted and repetitive behaviours of ASD (Atmaca et al., 2007). Post-mortem brain studies on pathology in the patients with autism have provided some evidence of abnormalities in neuronal regions including the cerebral cortex as well as a decreased number of Purkinje cells in the cerebellum (Garbett et al., 2008). The previous studies have detected an increased total cerebral grey and white matter, excess neurons in the pre-fontal cortex, and an increase in brain

weight at autopsy in the brain of children between two and four years old diagnosed with ASD (Courchesne et al., 2011, Hazlett et al., 2011).

1.2. Autism spectrum disorder

1.2.1. What is autism spectrum disorder?

Autism Spectrum Disorder (ASD) refers to a group of neurodevelopmental disorders including autistic disorder, Asperger's syndrome (AS) and pervasive developmental disorder-not otherwise specified (PDD-NOS). The new diagnostic criteria of ASD focuses on two core domains: social-communication impairment and restricted interests/repetitive behaviours (Sharma et al., 2018b).

ASD is characterized by very early onset before three years of age with a prevalence of one in 54 children according to Centers for Disease Control and Prevention (CDC) (Christensen et al., 2019). As a complex disorder, the phenotype and severity of autism differ for each patient. ASD affects social interaction and adaptability, which has serious implications for the development of the child (Masi et al., 2017). Eugen Bleuler coined the concept of "autism" in 1908 based on the study of the manifestation of severe schizophrenia patients (Maatz et al., 2015). In 1943, Leo Kanner conducted a study on 11 children. These children were characterized by difficulties in social communication, difficulties in adapting to changes in habits, good memory, sensitivity to stimuli (especially sound), resistance and food allergy, good potential intelligence, parody or tendency to repeat words of the others and also in spontaneous activity (Kanner, 1943). These findings are described in his paper entitled "Autism disturbance of Effective contract" (Henninger and Taylor, 2013). In 1944, Hans Asperger studied independently another group of children and they also had the same characteristics as Kanner's description. However, the children in his study displayed a problem of language and they did not copy words from others. He also said that many of these children were clumsy and different from normal children in motor skills (Asperger, 1944). The first epidemiological study of autism carried out by Victor Lotter reported that the prevalence was 4.5 in 10,000 children with an increased rate of 116.1 per

10,000 children in the United Kingdom (UK) - a figure that continues to rise (Evans, 2013). Such a prevalence increases the general awareness and focus on autism.

1.2.2. Clinical features

Each child with ASD seems to have an unique pattern of clinical symptoms and level of severity but they all share some common features described: Social communication and interaction: A child with ASD may have problems with social interaction and communication skills, for example: she/he does not respond to his/her name, lack of or poor eye contact, limited facial expression, feelings, emotions, they prefer to play alone, find difficulties to connect with peers, generally show delayed speech development and may use abnormal voice tone. Patterns of behavior: A child with ASD may have limited and repetitive patterns of behavior, a small field of interests or activities, engage in repetitive movements or specific routines, demonstrate odd movement patterns, tend to be fascinated by small details of an object, may engage in self-harm activities, may show sensitivity to light, sound or touch and often display picky food behavior. No single repetitive or self-injurious behavior seems to be specific to autism, but autism appears to have an elevated pattern of occurrence and severity of these behaviors.

1.2.2.1. Core symptoms

The symptoms of autism can be divided into 2 main categories: core symptoms and secondary/comorbid symptoms. The core symptoms include social deficits, communication impairment, and rigid ritualistic interests. Social deficits and communication impairment symptoms are suggested to merge into a single category of social-communication impairment, reducing the main symptoms of autism to social-communication impairment and rigid restricted interests. Although these symptoms could occur as signs in various psychiatric disorders, and sometimes over diagnosis is made, the presence of those in the same individual should be considered as an indication to carry out in-depth autism-specific evaluation. However, differential diagnosis still needs to be undertaken. In addition, the clinician should also consider the age of presentation because they may emerge at different periods of life. For example,

language deficits and hyperactivity are likely to present during early childhood in the children with autism while adolescents may show problems with relationships and regulation of mood. Besides the pattern of symptoms and the age of presentation, other factors including the existence of intellectual disability and any psychiatric disorders should be considered, as discussed later (Nazeer and Ghaziuddin, 2012).

1.2.2.2. Secondary or comorbid features

Nearly one half of autistic children present secondary features including hyperactivity, self-injurious behaviour (Kim et al., 2011, Dosreis et al., 2006) and these symptoms may significantly affect the quality of life of both patients and families. Depression, psychotic symptoms and suicidal behaviour may occur during adolescence (Nazeer and Ghaziuddin, 2012). Autism is a group of conditions characterized by impairments in socialization and communication, and repetitive interests and behaviors, but differ in developmental course, symptom pattern, and cognitive and language abilities. In addition to these three core features, the spectrum can include anxiety/attention deficit hyperactivity disorder (ADHD), cognition deficits, and medical co-morbidities (Figure 5).

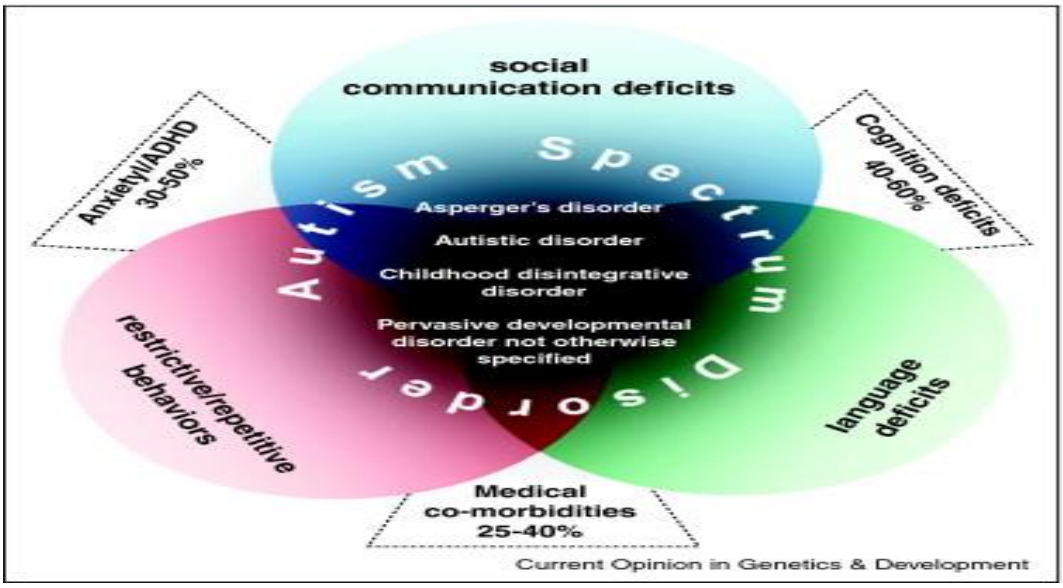

Figure 5: The main features of ASD (Devlin and Scherer, 2012)

1.2.2.3. Associated symptoms

1.2.2.3.1. Association with intellectual disability

Intellectual disability (ID), formerly referred to as mental retardation, is a neurodevelopmental disorder characterized by deficits in both intellectual functioning and adaptive behaviour (Lee K, 2021). (1) below-average intellectual functioning (also known as IQ) which is measured by an IQ test. The standard population typically mean scores is 100, while the ID patients are generally less than 70. (2) lack of adaptive behaviours (skills necessary for day-to-day life) (Boat TF, 2015). Studies have shown that a relatively high percentage of patients with autism suffer from intellectual disability (ID) and some ID patients also have autism (Srivastava and Schwartz, 2014). The relationship between autism and ID was the main reason to suggest that autism is a biological disorder rather than psychogenic condition caused by social problems. However, the understanding about any association between these disorders is not fully clear.

1.2.2.3.2. Association with epilepsy

Autism can co-occur with numerous other conditions with epilepsy as the most common. Some studies reported that nearly half of autistic patients have epilepsy: Viscidi et al., (2013) has shown that the average prevalence of epilepsy in nearly 6000 autism children aged 2-17 years was 12.5% and it raises to 26% if only counting aged 13 years and older (Viscidi et al., 2013). Other research in 7000 patients in 2019 found that 10% of ASD probands were also affected by epilepsy (Ewen et al., 2019). It has been suggested that people with epilepsy were also more likely than others to have autism. Sundelin et al., identified that of 85,000 people with epilepsy, those also affected by autism was ten times higher than the general population (Sundelin et al., 2016). Autistic patients seem to have most types of seizures and this disorder onset appears at two peaks: early childhood and adolescence. Many studies provide evidence that ASD and epilepsy share a common genetic origin with significant overlap between genes and other conditions linked to these (Novarino et al., 2013, Christensen et al., 2016).

1.2.2.3.3. Regressive Autism

It is estimated that the rate of regressive autism - a condition in which a child suddenly loses speech and social skills after a typical development - is around 20% in total autism cases (Wiggins et al., 2009). The children who suffered language loss also likely lose other communication skills. For example, a boy who used to be very talkative, then become very quiet and presents a pattern of losing nonverbal communication skills including imitation, eye contact, responding with his name. These symptoms are usually seen between the ages of 18 and 24 months (Kern et al., 2014). The loss of skills can be quick or slow and are usually followed by a stagnant skill period. With these cases, a history should be noticed such as infection or the emergence of seizures which may be the precipitant. But these are not always present as an obvious cause. Several studies reported that these children have a poorer prognosis (Goin-Kochel et al., 2017).

The statistically significant difference in regression concordance among dizygotic and monozygotic twins affected ASD provides evidences for the genetic contribution to regressive autism. The studies suggested that regressive ASD is a subtype of ASD and has potentially different causes (Tammimies, 2019). Goin-Kochel RP et al,. (2017) reported that some specific likely gene disrupting mutations have strong contribution to regressive autism and genes related to postsynaptic density proteins present a higher rates in regression (Goin-Kochel et al., 2017). There are around 35 out of over hundred ASD-related genes showed evidence with regressive ASD according to research (Tammimies, 2019).

1.2.3. When should autism be suspected?

ASD is a very complex, severe disorder and has many overlap symptoms with other psychiatric disorders. Therefore, a combination of appropriate instruments and scales could improve the quality of diagnosis that becomes important for clinical management of ASD patients. These assessment instruments are updating with better versions available in the last few years. Improved methods including parent/caregiver interviews, patient interviews, direct observation of patients, and family

history medical record of ASD and other neurodevelopmental disorders have become available. From a clinical point of view, the common signs that are present in these children are extreme shyness, socially awkwardness, language impairment or restriction in typical objectives or interests. Sometimes, other conditions such as language delay, severe hyperactivity, and obsessive-compulsive behaviour may present. They may also have intellectual disability or epilepsy. These symptoms are often noticed by parents or caregivers and children are then presented to the paediatrician and screened for ASD.

ASD could be classified as syndromic and non-syndromic ASD. Non-syndromic autism is a term applied for patients with autism is a primary diagnosis while syndromic autism is used to call a well-known condition caused by particular a genetic alteration, for example Rett syndrome, Fragile X syndrome, tuberous sclerosis (Ivanov et al., 2015). Patients with syndromic autism are not easily detected by clinical assessment because symptoms for example dysmorphic features, birth defects can vary widely and reported clinical information of each specific cases is still limited. These syndrome may be identified by genome wide tests including microarray, whole exome or whole genome sequencing (Fernandez and Scherer, 2017).

1.2.3.1. Mental status examination

ASD is typically characterized by very early onset, sometimes around a few months of age, but in regressive autism, the symptoms may be delayed. The screening and assessment of children with a suspected ASD has been studied for a number of decades and there are various assessment tools to evaluate autism patients of different ages. Every patient with any core features of ASD needs to perform a mental status examination (Boat TF, 2015). The core signs should be noticed including social-communication deficits (for example: poor eye contact, absence of social smiling, problems with personal boundaries, language abnormalities) and restricted interests (for example a tendency to fixate on certain topics or activities, compulsive behaviours and rituals). The medical specialists also need to evaluate level of hyperactivity, aggressive behaviours, self-injurious behaviours or suicidal behaviour.

In late adolescence and adulthood, the sign of regression particularly present as psychotic symptoms, alteration of speech or emergence of catatonia.

1.2.3.2. Physical examination

Physical examination of patients with autism is usually remarkable for repetitive behaviour, dyspraxia, abnormal gait and abnormality of motor functions. As mentioned above, ASD has a wide range of symptoms and severity and overlaps with other disorders, and so a brief medical examination is also needed to identify any other congenital abnormalities or dysmorphic features (such as increased head circumference) and include assessment of vision and hearing. These dysmorphism could be classified as major abnormalities (for example ventricular septal defect) or minor abnormality (such as low-ear set, single palmar creases). Miles et al,. (2005) suggested a physical examination used for affected children with physical anomalies including major and minor abnormalities to give a total score for each patient. These children were separated into 3 categories: essential (0-3), equivocal (4-5) or complex (>5). Patients with complex or equivocal score may show a genetical distinct from normal or essential ones (Fernandez and Scherer, 2017).

1.2.4. Classification and diagnostic criteria for ASD

The diagnosis of autism needs to combine details of developmental history and be the result of a systematic examination. Rating scales and questionnaire interviews that are well structured are useful for strengthening the process of clinical diagnosis. When ASD is complicated with severe intellectual disability or co-occurs with complex psychiatric disorders such as psychosis, the diagnosis should be made with caution since the assessment instruments may not be as accurate. The gold standard of autism diagnosis is the combination of information from systematic rating scales, structured interviews, observation schedules and overall clinical examination. The selection of assessment methods depends on the time and available resources. For a more accurate diagnosis, the medical team should at least

include a child psychiatrist, a speech and language therapist, a psychologist, and an educational consultant working together.

1.2.4.1. Using the Diagnostic and Statistical Manual (DSM)

In the last decades of the twentieth century, many debates took place around the definition and the diagnosis of autism. The psychiatric classificatory model was introduced in the Diagnostic and Statistical Manual (DSM), particularly *DSM-III* in 1980 (Evans, 2013). During the research of autism, scientists have come up with comprehensive diagnostic criteria in the American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorder (DSM) Autism Diagnostic (Kim et al., 2014). A number of modifications in the standards for ASD diagnosis have been reported in DSM-5, released in 2013. ASD includes autistic disorder, Asperger's Syndrome, childhood autism spectrum disorders, and pervasive developmental disorder not otherwise specified (PDD-NOS). Compared to DSM-4 diagnoses of autism, DSM-5 diagnosis no longer includes communication disorders as a separate criterion, and has incorporated social interaction and communication into the same evaluation criterion (Kim et al., 2014). Following DSM, autism patients would be classified to one of three levels of severity: **Level 3** - "Requiring very substantial support" (including severe social communication impairment in both verbal and nonverbal skills, very limited social interactions, minimal response to social overtures, inflexibility of behavior, very difficult to deal with change, extremely restricted/repetitive behaviors and interests), **Level 2 -** "Requiring substantial support" (including marked verbal and nonverbal social communication deficiency, limited social interations, reduced or abnormal responses to social overtures, difficult to deal with change, obvious restricted/repetitive behaviors and interests), **Level 1** - "Requiring support" (including only show social communication impairment in cases of no support, difficult to initiate social interactions, atypical or unsuccessful response to social overtures, difficult to switch activities or organise or plan independently).

1.2.4.2. Using the Childhood Autism Rating Scale (CARS) to distinguish between mid-to-moderate and severe Autism (Chlebowski et al., 2010)

CARS includes 15-item rating scales: Relationship to people; imitation; emotional response; body use; objective use; adaptation to change; listening response; visual response; taste, smell and touch response and use; fear and nervousness; verbal communication; non-verbal communication, activity level; level and consistency of intellectual response; and general impressions. Ratings are based not only on frequency of the behaviour in question, but also on its intensity, peculiarity, and duration. Each item is scored from one to four; higher scores are associated with a higher impairment level. Sum of rating values for 15 items produce a Total Raw Score from 15 to a high of 60 and scoring with under 30: non-autistic, 30-36: mild-moderate autism, 37-60: severe autism.

1.2.4.3. Autism Diagnostic Observation Schedule (ADOS) (Reaven et al., 2008)

Autism Diagnostic Observation Schedule (ADOS) is considered as a "gold standard" in autism diagnosis. The ADOS tool is a 60-minute interactive process between a child and a trained examiner. ADOS will provide direct information about the child's social interactions, language, behavior, use of toys, etc., which will provide evidence that helps to diagnose autism objectively and reliably. ADOS tool includes the organization of activities and use of toys designed to appeal and attract children. ADOS focuses on enabling children to communicate and comply with the requirements. During the implementation of ADOS, children will be prone to curiosity, interest in toys, use toys, play fantasy, have fun, looking at the eyes to communicate with the evaluator or parents. Children with autism spectrum are not able to or have great difficulty in expressing or showing usual communication skills in the process. ADOS tool is designed as four modules and the selection of an appropriate module is based on the developmental and language level of the child.

Denver, Autism screening technique (ASQ), and Modified checklist for autism in toddler-revised (M-CHAT) are other supportive tools for ASD diagnosis.

1.2.5. Associated syndromes

Syndromic autism combination between autistic symptoms and other specific malformations and/or dysmorphic features represents approximately 10% of all ASD cases (table 1) (Devlin and Scherer, 2012). These syndromes include Fragile X syndrome (mutations in *FMR1* gene), Rett syndrome (mutations in *MECP2* gene), tuberous sclerosis (TSC) (mutations in *TSC1* or *TSC2*) while other syndromes related to copy number variations such as Prader-Willi syndrome, Angelman syndrome and 15q11-13 duplication syndrome may be associated with autistic traits. Patients affected by a 22q11.2 deletion – (DiGeorge syndrome) and neurofibromatosis type 1 with behavioural changes are frequently seen in ASD population (Ornoy et al., 2016). Multiple other syndromes were also associated with ASD but cases are rare (Moss and Howlin, 2009). Many strategies have been suggested for the identification of genetic variants related to ASD. This could help in finding more syndromic forms of ASD which will also improve genetic links with phenotype (Ornoy et al., 2016). Therefore, clinical examination and genetic tests to determine the combination or overlapping symptoms amongst these syndromes will be very important for phenotype classification.

Table 1: Genetic syndromes associated with or predisposing to ASD

Genetic syndromes	**Gene/chromosome region**
Fragile X Syndrome	*FMR1*
Rett Syndrome	*MECP2*
Neurofibromatosis type 1	*NF1*
Tuberous sclerosis	*TSC1*, *TSC2*
Prader Willi Syndrome	Del paternal allele at 15q11–q13
Angelman Syndrome	Del/mutation in maternal *UBE3A*
Smith-Lemli-Opitz Syndrome	*DHCR7*

Smith-Magenis Syndrome	17p11.2 del
Velocardiofacial/DiGeorge Syndrome	22q11.2 del
ARX Syndrome	*ARX*
Untreated phenylketonuria	*PAH*
Cornelia de Lange Syndrome	*SMC3*
Ch 22q11 duplication syndrome	22q11.2 dup
Potocki-Lupski Syndrome	17p11.2 dup
Down Syndrome	Trisomy of chromosome 21

1.2.5.1. Fragile X syndrome

Individuals who have Fragile X syndrome have an increased rated of ASD. It is currently estimated that ASD occurs in 21-50% of males who affected with fragile X syndrome (Moss and Howlin, 2009). (Moss and Howlin, 2009, Wadell et al., 2013). Fragile X syndrome is the most frequent inherited cause of mental retardation and is a result of mutation in the Fragile Mental Retardation 1 (*FMR1*) gene located on the X chromosome. Typically the mutation is the result of an expansion of triplet repeats in the 5' untranslated region of the *FMR1* gene which prevents gene synthesis of the FMR protein (FMRP). FMRP is a RNA-binding protein that modulates mRNA trafficking, dendritic maturation and synaptic plasticity. The deficiency of this protein affects the regulation of other pathways including metabotropic glutamate receptors (mGluR1 and 5), aminobutyric acid A and B pathways, phosphatidylinositol 3-kinase, and mammalian target of rapamycin pathways. Structural brain network topology evaluation with MRI has found that the patients with Fragile X syndrome have deficits in global network organization and decreased clustering with connectomes. These abnormalities are similar to what has been found in ASD (Bruno et al., 2017). Newman et al., assessed 47 children and adolescents with Fragile X for ASD symptoms and found most of these patients were engaged in self

injury behaviour, aggression, and stereotypy (Newman et al., 2015). Patients who have both fragile X and ASD developed slower, had lower developmental level for age and worse language abilities than individuals who had only fragile X (Ballantyne and Núñez, 2016).

1.2.5.2. Rett syndrome

Rett syndrome is an X-linked disease which mostly affects girls. This disorder is characterized by neurodevelopmental delay, ASD and seizures. It is estimated that 25% - 40% of patients with Rett syndrome also have ASD (Moss and Howlin, 2009). Rett syndrome is caused by mutations in the methyl-CpG binding protein 2 gene *(MECP2)*. Methyl-CpG binding protein 2 is coded for by the *MECP2* gene and influences gene expression by binding to methylated-CpG dinucleotides. While MECP2 is expressed widely, it is especially high in neurons of the mature nervous system. The duplication in *MECP2* gene causes MeCP2 duplication syndrome with specific symptoms including autism, intellectual disability, motor dysfunction, anxiety, epilepsy, recurrent respiratory tract infections and early death. The MET receptor tyrosine kinase gene which contributes to the biological network that includes FOXP2 and MeCP2 (ASD-associated transcriptional regulators) was found reduced in the temporal lobe of subjects with ASD. Regulation of MET transcription by MeCP2 was found in vitro in the olfactory epithelium of human neural progenitors. Postmortem samples from temporal cortex of Rett syndrome patients and ASD patients were analyzed and demonstrated sex-based differences in reduced MET expression, with males' perturbation in ASD and female perturbation in Rett syndrome (Plummer et al., 2013).

1.2.5.3. Phelan–McDermid syndrome

Heterozygous mutations or deletions of *SHANK3* gene, which locates near the terminus of chromosome at 22q13 is related to Phelan–McDermid syndrome (PMS) and autism. Clinical features may include developmental delay with severely impaired language (> 75%), hypotonia (75%), and dysmorphic features (Sarasua et al., 2014). Autistic traits (84%), behavioral problems such as aggression (28%), and

seizures (27%) are commonly reported, and systemic involvement including gastrointestinal dysfunction (constipation 41%, gastroesophageal reflux 42%), renal anomalies (26%), and disrupted sleep (46%) (Soorya et al., 2013, Sarasua et al., 2014). *SHANK3* is one of the *SHANK* family members that organize a cytoskeleton-associated signaling complex at postsynaptic density. *SHANK3* combined with other genes codes for a scaffolding protein complex of the postsynaptic density of excitatory synapses, and *SHANK3* deficiency reduces synaptic transmission and plasticity. On this pathophysiological basis, the syndrome is considered to be a disease of synapses and is considered to be part of the synaptopathy-disease family. It is estimated that mutations in *SHANK3* are present in 0.69% of the patients with ASD (Leblond et al., 2014) and the prevalence of ASD in patients diagnosed with PMS had been reported with a wide range from 0 to 94% as the different obtained information (Philippe et al., 2008, Phelan and McDermid, 2012, Uchino and Waga, 2013). Interestingly, previous studies reported that PSM patients who meet criteria for ASD have smaller deletions only *SHANK3* gene while others who did not meet the criteria have larger deletions (more genes) with more severe phenotype (Sarasua et al., 2011, Phelan and McDermid, 2012). Therefore there are specific percentage of patients with PSM diagnosed with ASD. It is hard to distinguish these two disorders by clinical examinations only. MRI was applied to evaluate neural response to communicative vocal sounds and it showed the difference between infants with Phelan–McDermid syndrome and ASD. Those with Phelan–McDermid syndrome have better neural response to communicative vocal sounds in the right superior temporal gyrus (Wang et al., 2016a) while have less sensory sensitivity than those with ASD (Mieses et al., 2016). Therefore, it is useful to use genetic testing for the diagnosis of PSM in ASD patients.

1.2.6. Factors related to autism spectrum disorder

Biological research in autism has improved our understanding of the mechanisms of neurobiology that may be involved in ASD. Studies were conducted in varied areas such as genetics, neurochemistry, neurology, neuroscience, brain imaging and neuroimaging. In summary, the finding is autism cannot be

summarized or explained by a single biological factor, but rather by many causes (Vargas et al., 2005). Some authors support the hypothesis that the cause of autism is multicomponent interaction, and that environmental factors may interact with genetic factors that increase risk (Shen et al., 2010). Autism can be considered as a psychiatric illness because of the impact of diverse biological factors and/or psychological factors, including genetic, epigenetic, environmental factors and gene-environment interaction. Environmental factors may be both pre-natal or post-natal for example advanced paternal and maternal age at birth, gestational bleeding, multiple birth, medications, pollution, maternal depression and psychosocial and neurophysiological factors occurring during the postnatal period (Tordjman et al., 2014).

Images of the structure and function of the brain and neurophysiologic techniques applied in ASD have revealed abnormal growth - with hints of neuritis in the cerebral cortex of the forebrain and cerebellum, including activation of both neuroma and astrocytes. Magnetic Resonance Image (MRI) studies have shown many evidences for the implication of neurodevelopmental characteristics underlying ASD (Ecker et al., 2015). Various MRI results show abnormalities in both the grey and white matter with some brain regions in ASD compared to normal controls (Lange et al., 2015, Schumann et al., 2010). Specific core regions may relate to clinical phenotypes of ASD such as the frontotemporal lobe, frontoparietal cortex, amygdala, hippocampus, basal ganglia, and anterior cingulate cortex (Amaral et al., 2008). For example: (1) abnormalities in the inferior frontal gyrus (Broca's area), superior temporal sulcus, and Wernicke's area might be related to defects in social language processing and social attention (Redcay, 2008); (2) the frontal lobe, superior temporal cortex, parietal cortex, and amygdala might mediate impairments of social behaviours (Adolphs, 2001, Kim et al., 2010); and (3) the orbitofrontal cortex and caudate nucleus have been associated with restricted and repetitive behaviours of ASD (Atmaca et al., 2007). Morphologically, the brain may show no abnormalities on imaging such as Computed tomography (CT) or MRI. Positron emission tomography – computed tomography (PET-

CT) scan may however help in some of these cases. This type of scan detects cerebral blood flow and areas of hypoperfusion can be quantitatively mapped. The result from PET-CT can often be used to confirm the clinical diagnosis as well as for prognosis and post treatment follow up. Research has described the decrease of uptake value in PET-CT compared to standard uptake values in the hippocampus, amygdala, temporal lobes and frontal lobe (Chivate et al., 2016). Previous studies tried to use imaging techniques, genetic as well as transcriptomic approaches to clarify the pathogenic pathway of autism. Although there is conflict in the results, brain connectivity and synaptic function changes could often be seen in autism patients (Lord et al., 2000, Südhof, 2008). *NLGN1* (Neuroligin 1) and *NRXN1* (Neurexin) genes have been implicated as candidates for these alterations (Südhof, 2008) with an imbalance of neuronal excitation and inhibition potentially changing local and distal connectivity (Sporns et al., 2000, Zikopoulos and Barbas, 2013). The dysfunction of myelination pathways may also relate to these changes, as evidenced by the finding of circulating antibodies against myelin basic protein and myelin-associated glycoprotein in some autism patients (Zikopoulos and Barbas, 2013, Mostafa et al., 2008). Evidences could also be seen in neuroimaging and post-mortem pathology.

1.2.6.1. Neuroimaging

Neuroimaging studies of children with autism have detected abnormal brain overgrowth in various regions of the brain including prefrontal, temporal lobe and amygdala and abnormal functional asymmetry and activation in the cortex and cerebellum (Chow et al., 2012). Several neuroimaging techniques, for example functional MRI, were applied to reveal altered patterns of functional specialization in autism in domains of thinking, and visuospatial processing in children as well as adults with ASD (Maximo et al., 2014). Impaired functional connectivity may explain inefficiency in maximizing network connections to perform tasks, but over-connectivity does not mean greater

efficiency but may involve hyper-specialized reflection. Based on the abundance of information, inefficient connectivity may be a hallmark of ASD.

Several neuroimaging techniques including magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), positron emission tomography (PET), and functional MRI are applied to study the brain dysfunction in ASD. MRI was using in some structural and volumetric research that show the abnormalities in brain structure such as an increased brain volume, a decreased volume of vermian lobules VI and VII, a decreased volume of the amygdala, and an increased hippocampal volume were present in autism individuals. Other studies however, provided evidence that there was no difference in brain volume, the vermian lobules, amygdala and hippocampal in autism patients (Sharma et al., 2018a). PET is able to evaluate the functional abnormalities of the brain and (S, 1992) 2-fluoro-2-deoxy-D-glucose (FDG) is considered the most robust and longest half-life radiotracer. This is the most commonly used analogue in PET imaging because glucose is the main metabolic substrate in the brain (Zürcher et al., 2015). Previous studies using PET identified a decrease in blood flow in the temporal cortex as well as reduced glucose metabolism in both the thalamus and putamen and increased metabolism in both the occipital and parietal cortices (Sharma et al., 2018a). Currently though, there is no final conclusion of any specific metabolic pattern in autism patients' brains compared to control individuals.

1.2.6.2. Pathology

Post-mortem brain studies on pathology in the patients with autism have provided some evidence of abnormalities in neuronal regions including the cerebral cortex as well as a decreased number of Purkinje cells in the cerebellum (Garbett et al., 2008). The previous studies have detected an increased total cerebral grey and white matter, excess neurons in the pre-fontal cortex, and an increase in brain weight at autopsy in the brain of children between two and four years old diagnosed with ASD (Courchesne et al., 2011, Hazlett et al., 2011). In some autism patients, the rate of head growth and

brain growth is exceptionally rapid during the first few years of life. Additionally, although most children with autism are born normocephalic, during the first years of life, 15–20% will develop macrocephaly (Lainhart et al., 2006). In the later stages of life, neuron loss and cortical thinning have been observed with no enlargement in the brain by late adolescence and early adulthood (Kates et al., 2004).

1.3. Genomic contributions in Autism spectrum disorder

Chromosome is a long DNA molecule tightly coiled many times around proteins called histones that found in the nucleus of the cell. In human, each cell has 23 pairs of chromosomes, 22 autosomal chromosome and one sex chromosome.

Copy number variations (CNV) are the alterations in the structure of chromosome with at least 1Kb in size and involving the number of copies of specific regions of DNA.

Gene is a basic unit of heredity and a sequence of nucleotides. Some genes encode to the synthesis of protein while many others do not. In human genome, there are over 20,000 genes that could be vary in size from few hundred base pairs to millions. We have normally two copies of each gene, one inherited from each parent.

1.3.1. Heritability of autism spectrum disorder

Studies have shown evidence that ASD is a result of complex interactions from gene and environment, with heritability estimates ranging between 40% to 80% (Chaste and Leboyer, 2012). Hundreds of genes have been suggested to be associated with autism spectrum disorder (Rylaarsdam and Guemez-Gamboa, 2019). (Rylaarsdam and Guemez-Gamboa, 2019). Studies of twin pairs, high-risk infant siblings, families, and populations have estimated concordance rates and segregation of ASD within families. The very first twin study carried out by Folstein and Rutter in 1977 reported the concordance rate (the presence of the same trait in both members of a pair of twins) was 60% in monozygotic twins

compared to only 10% in dizygotic twins. Twin studies reported high heritability between 60-90% depending on the measure protocol and age of the participants (de Zeeuw et al., 2017, Ronald and Hoekstra, 2011). A meta-analysis of data from seven recent twin studies found the heritability of 64-91% (Tick et al., 2016). Results from a recent large prospective study has shown that the recurrence rate was 18% in infant siblings and 33% in multiplex families. It is currently estimated, that genetic variation contributes over 50% of the risk of developing ASD (Yoo, 2015). Since the 1990s, studies on the role genetics in autism have been undertaken but it still remains a challenge for geneticists. Autism as well as other complex diseases such as hypertension, diabetes, and heart disease are caused by multiple genes involving or interacting with each other. It involves a spectrum of mutations from single gene mutations to large chromosomal variations and even gene-environment interactions. The high heritability of ASD (50-80%) suggested that not only one model could explain for autism (Hallmayer et al., 2011). Multiple gene-disruptive events may co-occur in probands that lead to a more severe phenotype (Turner et al., 2016, Girirajan et al., 2010, Guo et al., 2019). Studying the genes involved in autism is indeed a challenge for geneticists around the world. Recent technological developments such as the study of the genome-wide associate study of single nucleotide polymorphisms (SNPs), the study of copy number variants – (CNV), the entire exome sequence, and sequencing of the entire genome have become effective tools in the study of complex diseases (Table 2) (Sener et al., 2016).

Table 2: Recent advances in the genetic studies of ASD (Sener et al., 2016)

Year	Methods	Related events
1943	Clinical exam	Identification of autism by LeoKanner (Kanner, 1943)
1970-	Twin studies	Report high heritability between 60-90% in monozygotic twins with no significant shared environmental contribution but only 10% in dizygotic twins that provides strong evidence of genetic

		contribution to the development of autism ((de Zeeuw et al., 2017, Ronald and Hoekstra, 2011, Tick et al., 2016).
1985-	Karyotype	Early karyotype studies started to shed light on related chromosomal regions including chromosomal abnormalities such as Down syndrome (trisomy 21), Klinefelter syndrome (47,XXY), Jacob syndrome (47,XYY) and structural chromosomal abnormalities including Prader Willi/Angelman syndrome region (Gillberg and Wahlström, 1985, Fernandez and Scherer, 2017).
2007-	Chromosomal Microarray	The first CNV studies on patients with ASD were published in 2004 (Iafrate et al., 2004, Sebat et al., 2004) and it was suggested that CNVs containing one or multiple genes can alter gene function. Overall, *de novo* CNVs are suggested to contribute to a significant proportion of the risk in autism with 5-15% of autism probands having *de novo* CNVs compared to only 1-2% of the general population (Geschwind and State, 2015) and until now, over 50 deletion/duplication syndrome related to autism phenotype have been reported (Iossifov et al., 2014, Iossifov et al., 2012, Ziats et al., 2021).
2009-	NGS Whole exome sequencing Whole genome sequencing	Whole exome and genome sequencing help to investigate genetic variants and their specific type (non-sense, missense,…) in autism patients, especially trio based exome sequencing is a powerful approach for identifying new candidate genes for ASD (separate into *de novo* and inherited variants). The potential related biological

		pathways from these genes could be predicted by the help of bioinformatic tools. Until now, hundreds of ASD risk genes have been identified (Rylaarsdam and Guemez-Gamboa, 2019, Satterstrom et al., 2020, Dias and Walsh, 2020).

Estimations of autism heritability have varied substantially depending on the examined population and methodology. Simplex family studies focus on de novo variants, while multiplex families studies show enrichment for inherited factors (Sener et al., 2016). Reports of identification of other family members of probands also affected by autistic traits or autism spectrum phenotype has been proven very useful towards genetic features in ASD (Wheelwright et al., 2010). Additional evidence supporting an underlying genetic cause is the higher relative risk for other psychiatric conditions in family members of probands compared to the general population (Constantino et al., 2010, Frazier et al., 2015, Jokiranta-Olkoniemi et al., 2016). A few studies have indicated that increased paternal age may also be related to an increased risk of autism due to *de novo* mutations in the sperm (Frans et al., 2013, McGrath et al., 2014, Sandin et al., 2016, Gratten et al., 2014, Kong et al., 2012).

Early karyotype studies started to shed light on related chromosomal regions (Gillberg and Wahlström, 1985). Karyotype test could detect chromosomal abnormality in 2% of ASD cases including 15q region related to Prader-Willi/Angelman syndrome, Down syndrome, sex chromosome aneuploidies (Miles et al., 2005, Miles, 2015, Wassink et al., 2001). For example, 5-15% of Down syndrome patients meet ASD criteria. While most CNVs and mutations which relate to ASD detected by genome-wide approaches (Fernandez and Scherer, 2017). Powered by the use of high-throughput techniques – microarray-based comparative genomic hybridization (aCGH) or chromosome array (CMAs) and more recently the whole exome and whole genome sequencing, many more genetic alterations have been

detected amongst the autism population (Sener et al., 2016). A rich body of literature provided evidence of de novo copy number variants contribution to the development of autism. These variants account for a largest contributors to known autism risk (Turner et al., 2017, Brandler et al., 2016, Aguet et al., 2017, Dias and Walsh, 2020). Until now, over 50 deletion/duplication syndrome related to autism phenotype have been reported but understanding of the mechanism of pathogenicity is still challenging (Iossifov et al., 2014, Iossifov et al., 2012, Ziats et al., 2021). The exome or genome sequencing revealed the important contribution of de novo single-nucleotide variants (SNVs) to autism risk that improves our understanding of the underlying neurobiology. There are hundreds genes related to autism have been reported (Rylaarsdam and Guemez-Gamboa, 2019). Loss of function variants provide powerful evidence about the biological network altered in autism (De Rubeis et al., 2014, Kosmicki et al., 2017). For example, the biggest exome sequencing studies on 35,584 people with 11,986 autism patients from both family-based and case-control studies applied enhanced bioinformatic analysis identified more than 100 autism-associated genes with a false discovery rate equal or less than 0.1 (Satterstrom et al., 2020). Genetic causes of autism spectrum disorders can be divided as follows: ASD-related syndromes (~10%), rare chromosomal abnormalities (~5%), rare copy number variant (~5%), rare penetrant genes (~5%), leaving 75-80% still of unknown cause (figure 6) (Devlin and Scherer, 2012).

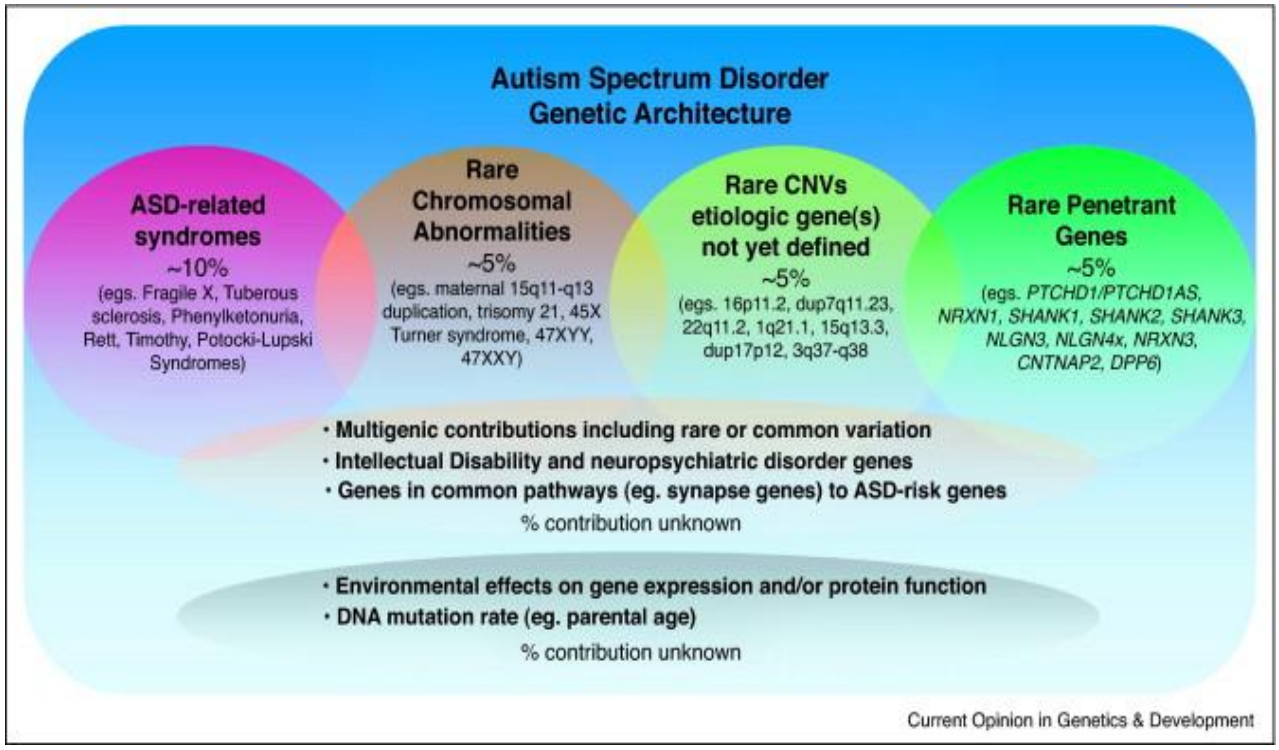

Figure 6: Autism spectrum disorder genetic architecture (Devlin and Scherer, 2012)

1.3.2. Genetic heterogeneity and clinical phenotypes

Genetic studies on autism patients in the last decade yielded a large amount of data, including CNVs, to support the exploration of the functional significance and relevance of genomic variation to the clinical manifestation of ASD. Researchers have attempted evaluating the importance of CNVs to the contribution of particular clinical features in ASD patients. The collected evidences show that ASD patients with causative CNVs exhibit more severe symptoms and deficits in language skills than individuals with non-causative CNVs or without CNVs (Robinson et al., 2014). Other research suggested that CNVs also have an influence on phenotypic diversity. A study found that intellectual quotient (IQ) has a relevance to the number of genes related in rare *de novo* CNVs only in males and also to the severity of ASD (Robinson et al., 2014). Bishop et al., (2008) used data from the Simons Simplex Collection to determine that individuals who have *de novo* events show greater motor delay and relatively preserved language abilities (Bishop et al., 2008). Merikangas et al. (2015) studied 1590 ASD patients and reported that CNVs previously implicated in ASD/intellectual disability were linked

to communication and language delay while CNVs which contain differentially brain expressed genes were associated with the manifestations of adaptive function (Merikangas et al., 2015). Yuen et al. (2017) used the data from the MSSNG resource of multiplex families to highlight that siblings having different mutations had a bigger variability compared to those who exhibit the same mutation (RK et al., 2017). This study also reported that probands who have mutation in ASD-risk genes had lower adaptive abilities than those without mutations. Although, some valuable data showed genotype-phenotype correlation, more studies are required to investigate the association between particular variants and specific features in adaptive abilities or in manifestation of symptoms in ASD.

1.3.3. Copy number variations and autism spectrum disorder

1.3.3.1. Copy number variation

CNVs are common in human genome and contribute to the biodiversity of the human. It is estimated that 95% of CNVs are "common variants" with the frequency of > 5% in the general population while only around 1% mostly occur in single individuals or families and are classified as rare CNVs (Bourgeron, 2016). CNVs could be classified as inherited or *de novo* (Thapar and Cooper, 2013). *De novo* CNVs are more prevalent in sporadic genomic disorders and are potentially related to a pathogenic phenotype by dysregulating gene expression (Vicari 2019). CNVs can be either deletion or duplication events. Deletions could be heterozygous (missing one copy of the usual two), homozygous (missing both copies) or hemizygous (deletions a part of X-chromosome in a male patient). CNVs can change gene dosage, interrupt coding sequences or even influence neighbouring gene regulation that subsequently has an influence on gene expression and phenotype. Sometimes they can cause exon shuffling (Sener, 2014).

1.3.3.2. CNV and detection methods

The many techniques used to detect specific CNVs include: FISH, Real-time PCR, MLPA, Sequenome Mass Array. Genome wide methods are widely using for CNV detection because of these advantages

including high throughput, high resolution, time and expense saving (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6128388/). Genome-wide CNV detection methods are classified into two main types: (1) array based (aCGH and SNP genotyping) and (2) next generation sequencing based.

1.3.3.2.1. Array based methods

Array based comparative genome hybridization (aCGH) method is now used as the most popular detection method for CNVs. It uses differentially fluorescence labelled test and control subject DNA co-hybridized on a slide having up to millions of probes representing matched genomic locations. This method measures comparative fluorescence between the test DNA and a control DNA. Deviation of fluorescence in test sample DNA from control DNA indicates presence of CNV with a duplication CNV is defined as an increased of fluorescence compared with baseline whereas a fluorescence ratio below baseline is considered an indication of deletion in the test DNA (see figure 7).

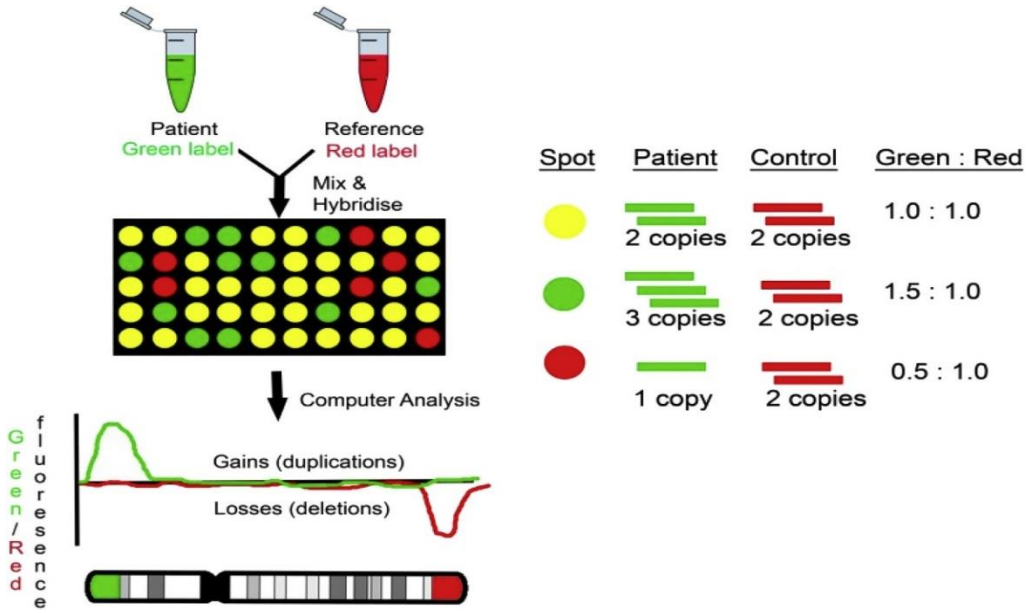

Figure 7: Diagram of microarray comparative genomic hybridization (Stosic et al., 2018)

SNP genotyping is another modification of the array-based technique. This DNA microarray is a powerful tool for high-throughput by using hundreds of thousands to millions of SNPs in a single experiment. The probes spotted onto the array are sequences flanking a known SNP. Affymetrix and

Illumina are two companies that provide the most popular SNP array kit commercially. While these arrays are usually made for genomic scale CNV detection they can be modified for any particular genomic location for detailed or fine CNV detection. Increased or decreased fluorescence measurements relative to other probes are taken to indicate duplications or deletions on these arrays (Feuk et al., 2006b). This method does not utilise co-hybridization of reference DNA on the array. The fluorescence value of each sample DNA is normalized with baseline or ploidy of two, calculated from pooled control individuals' DNA representing a diploid genome. Compared to other techniques, for example qPCR and MLPA, SNP array based methods can detect CNVs with a much higher validation rate (>80%) (Iafrate et al., 2004). There is a limitation with this technique as this method only detects change in DNA copy and cannot detect inversions or balanced translocations where the amount of DNA is same in an individual and the control. These arrays also cannot identify the size of duplication, only the presence of a positive copy number change in the genome complement (see figure 8).

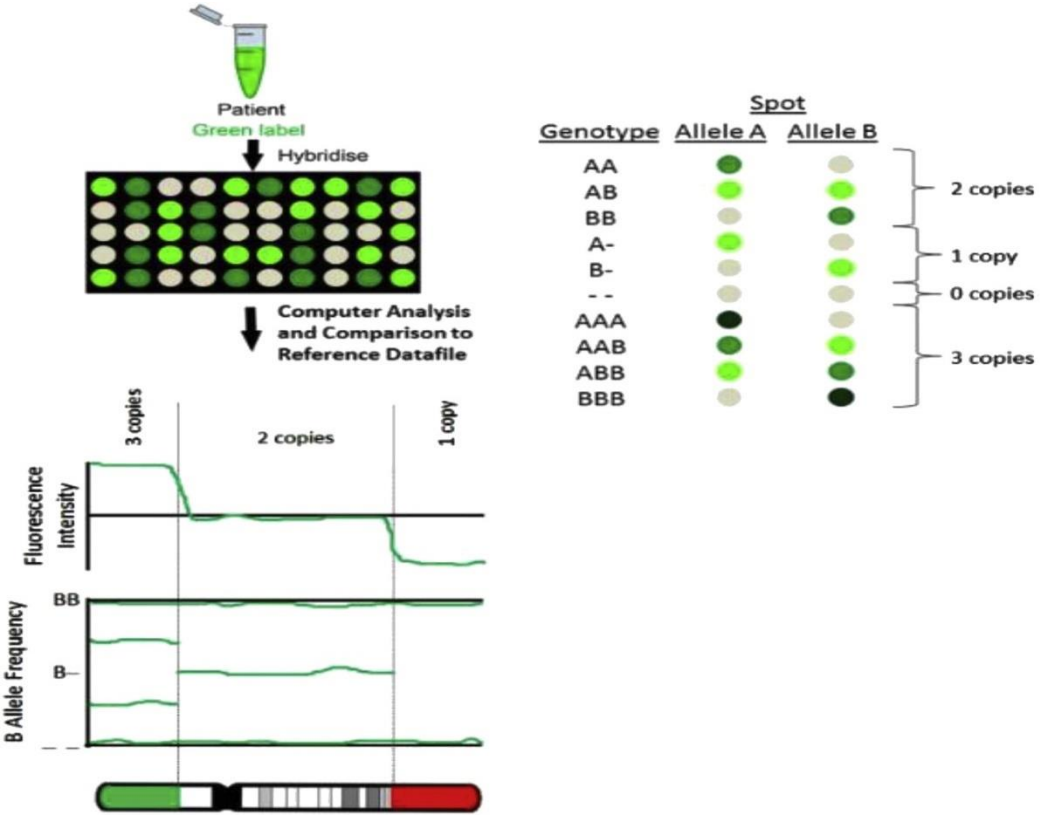

Figure 8: Diagram of SNP array (Stosic et al., 2018)

1.3.3.2.2. Next generation sequencing based CNV detection

In recent years, next generation sequencing (NGS) has been applied to genotyping, including comprehensive characterization of CNVs, by using millions of short reads. The advantages of the NGS-based approach compared to array systems are the higher coverage and resolution, more accurate estimation, ability for breakpoint detection and higher capability for novel CNV identification. NGS-based CNV detection methods can be classified into five categories including (1) paired-end mapping, (2) split read, (3) read depth, (4) *de novo* assembly of a genome and (5) combination of the above approaches. Each of them have their own advantages and limitations (Zhao et al., 2013). Figure 9 provides the basic information about these approaches.

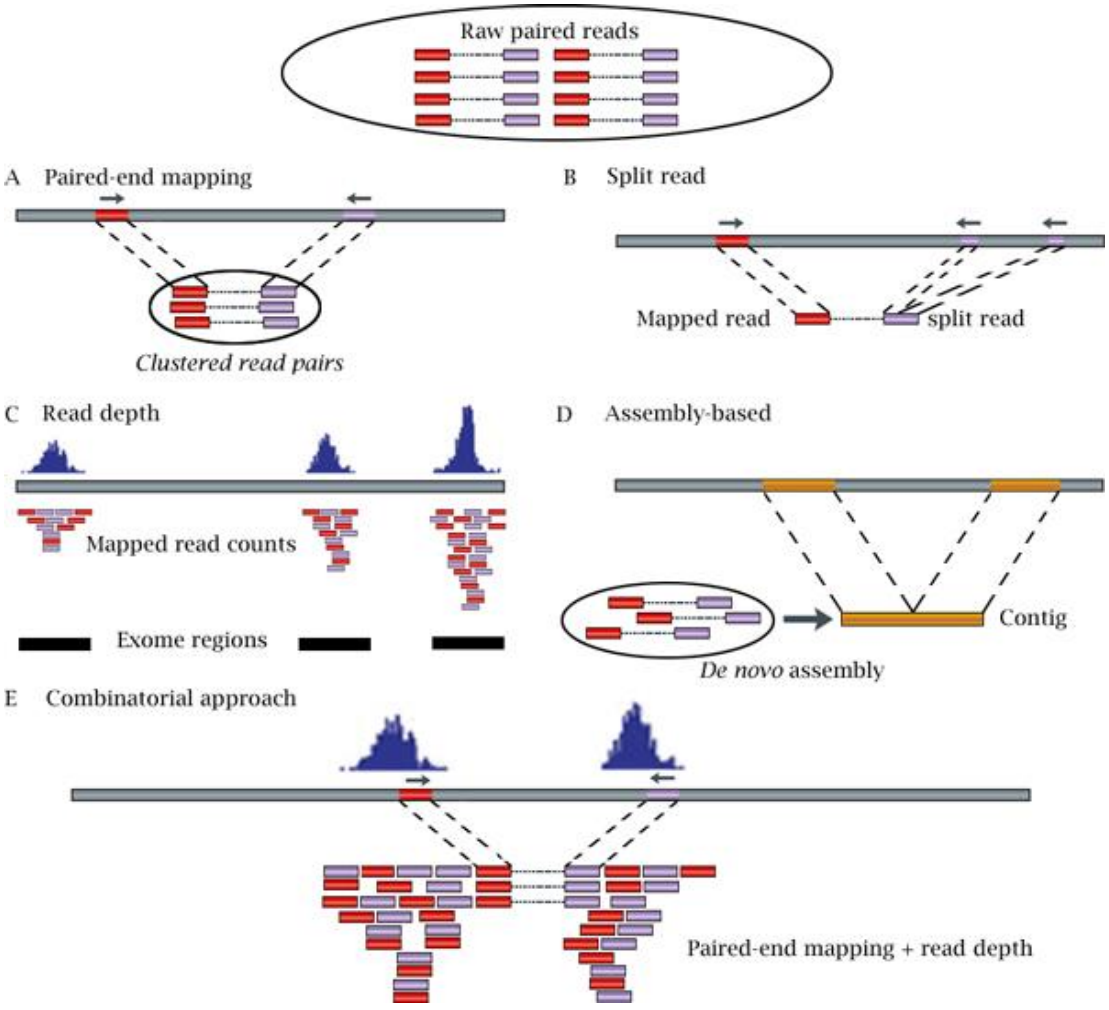

Figure 9: Five approaches to detect CNVs from NGS short reads (Zhao et al., 2013)

1.3.3.2.3. State-of-the-art method for CNV detection

aCGH has served as a robust and effective method for CNV detection (Feuk et al., 2006a). Well-developed data analysis methods are readily available and reported as many article review (Wineinger et al., 2008, Medvedev et al., 2009). Due to the high repetitive of genome, GC-content bias and low-coverage-depth data, sequencing error the false-positive rate of CNV calling is still not easy to control (Zhao et al., 2020). There are a very limited studies applied NGS for the identification of chromosomal aneuploidies and rearrangement. The current gold standard method for detecting copy number variants including microdeletions/duplications is aCGH (Russo et al., 2014). CMA is confirmed as a first tier test for diagnosis for individuals with developmental delay, intellectual disability, autism spectrum disorder and dysmorphic features (Battaglia et al., 2013).

1.3.3.3. CNV classification

Many recurrent CNVs are well characterized while most CNVs need further investigation for detecting their potential clinical significance. It is still challenging due to absence, limitation or conflicting genotype-phenotype correlation. The American College of Medical Genetics and Genomics (ACMG) and the National Institutes of Health (NIH)-funded Clinical Genome Resource (ClinGen) collaborated for updating a guideline for professional clinical laboratory practice standards for evaluating CNVs. They built a scoring metric to evaluate CNVs. There are five categories: pathogenic, likely pathogenic, uncertain significance, likely benign and benign (Riggs et al., 2020).

Pathogenic CNVs: 0.99 points or higher score using the evidence scoring metric.

Pathogenic CNVs may include: (1) reported in multiple peer-reviewed publications for consistent clinical phenotype; (2) overlap completely with an established dosage sensitive region; and (3) contain at least one gene with well-known dosage sensitive. This category will include most cytogenetically visible alterations (generally >5 Mb).

Likely pathogenic CNVs: score between 0.90 and 0.98 points.

These CNVs have strong evidence for disease-causing but not enough for definitive pathogenicity.

Likely pathogenic CNVs may include: (1) deletions involving the 5’ end (plus additional coding sequence) of established haploinsufficient (HI) genes (in scenarios where there are no known alternative start sites) (category 2C-1, deletion metric); (2) deletions involving multiple exons (through the 3’ end of the gene) in an established HI gene (category 2D-4); and (3) deletions or duplications involving genes with multiple case reports reported in consistent, highly specific phenotypes.

Variants of uncertain significance (VUS): score between −0.89 and 0.89 points.

They may include: (1) contain no gene in the affected genomic interval; (2) present in few cases in general population but still <1% to be called a polymorphism; (3) contain genes with unknown dosage sensitive; (4) no conclusion regarding clinical significance in multiple publications; and (5) contain gene with an unclear effect on the transcript reading frame.

Likely benign: score between −0.90 and −0.98 points.

These CNVs have strong evidence for being not involved in disease but not enough for definitively conclude. They may include: (1) no statistically significant difference between observations in cases and controls; and (2) frequently present in the general population but lower than 1%.

Benign: score −0.99 or fewer.

These CNVs have been reported as benign in multiple peer-reviewed publications or databases or represented a common polymorphism (>1% in the population).

1.3.3.4. Databases

1.3.3.4.1. Database of Genomic Variants (DGV)

The Database of Genomic Variants (DGV) collects data from the publication of the inaugural CNV articles that involved the genome wide CNVs of the healthy population and clinically unaffected individuals. At the start, the database contained data from only a few hundred individuals with around 1000 CNVs and some inversions but has now expanded to 2.5 million entries from 55 studies. Early

results were mainly generated using low-resolution microarrays with only a few samples and were limited by high false-positive and false-negative rates. This has resulted in a number of these CNVs subsequently being removed from the database after a curation process. Currently, data collected from high resolution microarray and genome sequencing by NGS have begun to populate DGV which has given a significant improvement in the accuracy of the database (MacDonald et al., 2014).

DGV aims to catalogue the highest quality structural variants collected from the literature and is formally accessible by both researchers and clinicians. It also possible to check the data through tracks displayed on the University of California, Santa Cruz Genome Browser (UCSC). UCSC Genome Browser is an online and downloadable database hosted by University of California, Santa Cruz. The database links to a variety of genome analysis tools that help to quickly and easily access to the data and annotate at many levels.

1.3.3.4.2. Simons Foundation Autism Research Initiative (SFARI)

SFARI was launched in 2006 from the Simons Foundation's suite of programs. Its mission is to improve the understanding of ASD in terms of diagnosis and enable treatment of the highest quality. Now, there are over 2000 CNV loci and 913 genes with 363 high-confidence and strong candidate autism risk genes that have so far been identified and are listed in SFARI database.

1.3.3.4.3. Other databases

Variants can also be checked for extra information via the International Standards for Cytogenomic Arrays (ISCA) dosage sensitivity map, Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER). The genes inside any CNV can also be checked in the Online Mendelian Inheritance in Man (OMIM).

1.3.3.5. CNVs in autism spectrum disorder

Although studies have convincingly demonstrated an apparent strong genetic contribution to ASD, it is still difficult to pinpoint the responsible genetic variants. After two decades of research, genetic

heterogeneity with multiple modes of inheritance has been shown including rare monogenic causes with partial penetrance, common variants of small effect, and inherited and *de novo* CNVs that impacts multiple genes (Chen et al., 2015).

The first CNV studies on patients with ASD were published in 2004 (Iafrate et al., 2004, Sebat et al., 2004) and it was suggested that CNVs containing one or multiple genes can alter gene function by "(1) disrupting the coding region as a recessive or dominant allele, (2) disrupting the regulatory landscape, (3) generating a chimeric gene or by (4) position effect" (Cook and Scherer, 2008). Studies have shown the contribution of cytogenetically visible chromosomal anomalies is approximately 7- 8% in the autistic population (Iourov et al., 2008). The development of the high resolution technique of chromosome microarrays (CMA), overcame the limitation of conventional cytogenetics and allowed scanning for both microscopic and sub-microscopic chromosomal aberrations in the whole genome (Zahir and Friedman, 2007). One genetic cause of ASD are *de novo* variants that originate from mutations in the parental germ line or early somatic cells during the development of the body. They play an important role in the genetic contribution to ASD due to their large target size, reduced fecundity, and high prevalence. In 2007, Sebat et al reported the first family-based study on CNVs by recruiting 118 simplex, 47 multiplex, and 99 control families. They found 10% patients had *de novo* CNVs compared to 3% in multiplex families and 1% in controls (Sebat et al., 2007). A Study had found that the rate of de novo CNV in affected individuals was 4 fold higher than unaffected ones (Itsara et al., 2010). Girirajan et al reported that the increase in global CNV load is mainly due to *de novo* events in the affected child (Girirajan et al., 2013). Further, female probands were observed to carry more *de novo* CNVs than male probands (Sanders et al., 2015). In addition, it is reported in multiple studies that a greater number of *de novo* CNVs was related to lower IQ (Leppa et al., 2016). Overall, *de novo* CNVs are suggested to contribute to a significant proportion of the risk in autism with 5-15% of autism

probands having *de novo* CNVs compared to only 1-2% of the general population (Geschwind and State, 2015).

Autism databases from related studies identified many different loci on various chromosomes suggesting multiple genes are involved in the development of ASD and suggesting the possible genetic contribution of CNVs to the pathogenesis of ASD.

By summarizing the findings of many of studies concluded that:

(1) Multiple rare *de novo* (and some inherited) CNVs contribute to ASD susceptibility. All have an individual contribution of less than 1% in the frequency of occurrence. (Sebat et al., 2007, Marshall et al., 2008, Weiss et al., 2008, Kumar et al., 2008, Glessner et al., 2009).

(2) The proportion of *de novo* CNVs is three to five times higher in families with ASD than in controls, and it is higher in simplex than multiplex families (Sebat et al., 2007, Marshall et al., 2008, Kumar et al., 2008, Weiss et al., 2008).

(3) Several patients with ASD have two or more *de novo* CNVs and typically have more severe phenotype. Approximately 27% patients with syndromic ASD have *de novo* CNVs (Weiss et al., 2008, Kumar et al., 2008).

(4) Up to 40% of family-specific CNVs are inherited from an apparently non-ASD parent, suggesting incomplete penetrance (Jacquemont et al., 2006, Sebat et al., 2007, Marshall et al., 2008).

(5) Although many CNVs appear to involve haploinsufficient regions, some act recessively as homozygous alleles deleting both copies of a gene in consanguineous families (e.g., *PCDH10, DIA1, NHE926*) (Morrow et al., 2008).

(6) Some CNVs recur at the same locus among unrelated patients and may coincide with genomic disorders associated with ASD. Some are well-known (e.g., maternal 15q11-q13 duplication) while the others are more recent findings (e.g. 16p11.2 deletion/duplication) and 17p11.2 duplication

(Jacquemont et al., 2006, Szatmari et al., 2007, Christian et al., 2008, Weiss et al., 2008, Glessner et al., 2009).

(7) There is a relative enrichment within CNVs in ASD studies for neuronal synaptic complex genes (e.g., *SHANK2, SHANK3, NRXN1, and NLGN4*) (Jacquemont et al., 2006, Sebat et al., 2007, Marshall et al., 2008, Morrow et al., 2008, Weiss et al., 2008, Glessner et al., 2009).

(8) CNV studies in ASD identified the same genes spanning across different neuropsychiatric and neurodevelopmental disorders including ADHD, SZ and ID, indicating that overlapping pathways may be involved in phenotypically distinct outcomes (Marshall and Scherer, 2012).

An important thing to remember is the penetrance for ASD depends on multiple factors including dosage sensitivity and function of gene(s) contained in CNVs. For example, CNVs that are *de novo*, is higher in the frequency of occurrence and associated with more severe symptoms will typically have a larger impact on ASD susceptibility. Some with moderate or mild effects require a "second-hit" or "multiple-hits" for crossing to phenotype threshold (Chung et al., 2014). And compared to duplications, deletion CNVs seem to have a stronger contribution to the severity of neurodevelopmental diseases (Coe et al., 2012).

Recent studies have shown that the spectrum of ASD is diverse, occurring in many different chromosomal regions (Table 3).

Table 3: Top CNV loci for ASD susceptibility

Genomic loci	**CNV aberration**	**OMIM**
1q21.1	Deletion	#612474
1q21.1	Duplication	#612475
2p16.3	Deletion	#614332
7q11.23	Duplication	#609757
15q11.2	Deletion	#615656

15q13.3	Deletion	#612001
15q11q13	Duplication	#608636
16p11.2	Deletion	#611913
16p11.2	Duplication	#614671
22q11.21	Duplication	#608363
22q11.2 distal	Deletion	#611867
22q13.33	Duplication	#615538

1.3.4. Candidate genes in autism spectrum disorder

Based on studies related to autism spectrum disorder and SFARI – the biggest database on autism, it is estimated that over a thousand genes have been proposed as candidates linked to ASD, but around 400 genes have enough evidence to be considered as true autism genes and only *de novo* and transmitted loss of function mutations could directly contribute to the disorder. The different genetic variant types, patterns of inheritance or *de novo* origin may define the potential risk of this mutation for the development of autism. For example, carrying a *de novo* SNV and a specific nonsense mutation in the coding sequence of the remaining gene confers around five times more individual risk than carrying a transmitted CNV alone (Stein et al., 2013).The high confidence ASD genes for autism follow three main pathways: chromatin remodelling, transcription and splicing.

1.3.4.1. *SHANK* family

Shank (also known as ProSAP) proteins make up a family of scaffold proteins through their interaction with more than 30 synaptic proteins (Sala et al., 2015). This connection is essential for synaptic formation, glutamate receptor trafficking and neuronal signalling (Monteiro and Feng, 2017). SHANK proteins encoded by three genes *SHANK1*, *SHANK2*, and *SHANK3*, are strongly related to ASD (Guilmatre et al., 2014).

SHANK3 gene (OMIM: 606230) which is located on chromosome 22 has 22 exons that codify for a number of mRNA and protein isoforms. *SHANK3* gene encodes a protein that is found in many tissues of the body but most abundantly in the brain. This protein plays a role in the functioning of synapses where nerve cells (neurons) connect to each other at the cell-to-cell communication point. Within synapses, the SHANK3 protein acts as a scaffold that supports the sending of signals from one neuron to another. It also related to the formation and maturation of dendritic spines which are essential for the transmission of nerve impulses. Health conditions related to *SHANK3* mutation include 22q13.3 deletion syndrome (Phelan-McDermid syndrome), and ASD (Costales and Kolevzon, 2015). Deletion of a part of chromosome 22 at position q13.3 causes 22q13.3 deletion syndrome that is also called Phelan-McDermid syndrome. This region contains many genes including the *SHANK3* gene. As the result of deletion, one of the usual two copies of the *SHANK3* gene is missing and this results in a reduction of the amount of *SHANK3* protein to give the characteristic signs and symptoms of this syndrome. The *SHANK3* deficiency which decreases functioning of synapses and cell-to-cell communication between neurons is believed to contribute to the developmental delay, intellectual disability and absent or severely delayed speech (Costales and Kolevzon, 2015). *SHANK3* gene mutations also have been found in ASD patients. Most of these mutations reduces the amount of SHANK3 protein or alters the SHANK3 protein function that then disrupts the communication between neurons (Bucher et al., 2021).This may be the main contribution to ASD.

The *SHANK2* gene is located on chromosome 11 at 11q13.3 and has 25 exons with three alternative promoters and one stop codon that create 4 different isoforms. *SHANK2* gene encodes one of the SHANK family proteins that have functions in postsynaptic density. SHANK2 mRNA is strongly expressed in the brain including hippocampus, cortex, and cerebellum (Sala et al., 2015). Studies showed the correlation between *SHANK2* and ASD and ID (Zaslavsky et al., 2019). *SHANK2* deletions are related to moderated ID, developmental delay, and mild motor problems (Sala et al., 2015).

1.3.4.2. *GABRB3* gene

The *GABRB3* gene which contains ten exons with alternative splicing that codes for many protein isoforms is located at chromosome 15q12 in a cluster with 2 other genes encoding subunits of the ligand-gated ionic channel family (Glatt et al., 1997; Urak et al., 2006). This channel family serves as the receptor for gamma-aminobutyric acid, a major inhibitory neurotransmitter of the mammalian nervous system. It plays an important role in the formation of functional inhibitory GABAergic synapses in addition to mediating synaptic inhibition as a GABA-gated ion channel. The beta-3 subunits is expressed at different levels in the brain including cerebral cortex, hippocampus, cerebellum, and thalamus at different points of development and maturity. Alteration in this gene may be related to the pathogenesis of several disorders including Angelman syndrome, Prader-Willi syndrome, non-syndromic orofacial clefts, epilepsy and autism (Hammer et al., 2015).

1.3.5. Genetic pathways to ASD

ASD can be associated with either single-gene deficits or large chromosomal aberrations that suggests a multiple of genetic pathways for this disorder. It is believed that ASD represents a collection of disorders with a complex genetic etiology including one gene, multiple genes and gene-environment interactions. Chen et al (2015) collected data from many human genetic studies, neuropathology, mouse models and biological networks and then identified a model “many genes, common paths” for the development of ASD (Chen et al., 2015). They found that early deficits of neurodevelopmental and synaptic function may cause abnormal development of cortical layers. Hundreds of candidate genes contribute to the basic cell functions such as chromatin remodelling, metabolism and mRNA translation - all of which may affect neuronal processes including neurogenesis, neuron migration, axon guidance, dendrite outgrowth and synaptic formation and function (Gilbert and Man, 2017). Synapses are the fundamental units of brain communication through cell-cell junctions. ASD is an early onset disorder with the diagnosis before 3 years of age, a period during which intense synaptogenesis is happening

(Huttenlocher and Dabholkar, 1997, Guang et al., 2018). Many genes, such as *NRXN*, *NLGN*, *SHANK*, *TSC1/2*, *FMR1*, and *MECP2*, found as the potential causes of ASD converge on common cellular pathways that converge at the synapse (Wang et al., 2016b, Stessman et al., 2017). ASD patients often have a higher global burden of rare, large CNVs related to associate synaptic genes (Guo et al., 2017). These genes encode cell adhesion molecules, scaffolding proteins, and proteins involved in synaptic transcription, protein synthesis and degradation. Therefore, alteration of these may affect various aspects of synapsis functions like synapse formation and elimination, synaptic transmission and synaptic plasticity. This suggests that synaptic dysfunction may contribute at least in part to the pathogenesis of ASD in term of functional and cognitive impairment. Gene mutations and several environmental factors may cause synaptic impairments that are implicated in many other neurodevelopmental diseases including epilepsy, ID, developmental delay and attention deficit-hyperactivity disorder (ADHD). Other studies of the synaptopathology of monogenic diseases such as fragile X syndrome, tuberous sclerosis complex and Angelman syndrome also provide information for the high comorbidity with ASD.

1.3.6. Functional relationship networks inform ASD gene prediction

While the evidence of a possible genetic contribution to autism is not debatable, our understanding of the genetic aetiology of this disorder is still limited. The complexity of ASD points to the dysregulation of not one, but multiple pathways and biological processes. Network-biology studies have proven useful in refining our understanding of the molecular basis of ASD. Network analysis and pathway enrichment tools applied to "system biology" studies identify clusters of highly interconnected mutated genes related to the chromatin-binding process, the Wnt-B-catenin pathway and calcium signalling (Medina et al., 2018). More recently, machine learning based methods have used gene interaction networks (Krishnan and Zhang, 2016) and cell-specific expression profiles (Zhang and Shen, 2017) to predict

gene involvement in autism. Importantly, the results of these studies lead to a quantitative metric that scores every gene in the genome according to evidence of a possible role in autism.

1.3.7. Genetic screening and counselling for ASD patients and their family

1.3.7.1. Genetic testing

Genetic screening is a powerful method for detecting causes of monogenic Mendelian disorders as having a direct genotype–phenotype correlation but with complex disorders such as ASD, it seems to be less useful. However, it still has value in terms of two aspects: evaluating the degree of genetic susceptibility to a certain disease and to identify rare monogenic or cytogenetic forms of the disease. During several decades of research in ASD patients, important successes were achieved including the identification of several vulnerability loci and of a few cytogenetic abnormalities or single-base mutations able to cause autism. Autism could be divided into syndromic autism (ASD associated with a known cause) and "idiopathic" or "primary" ASD. Syndromic autism associated with well-known genetic or genomic disorders such as fragile X syndrome, neurofibromatosis, tuberous sclerosis, untreated phenylketonuria, and Angelman, Cornelia de Lange and Down syndromes account about 4-5% of all ASD cases (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5789213/). Studies reported the correlation between mutation or deletion of *SHANK3* gene and ASD with severe verbal and social deficits. Therefore, ASD individuals who show severe language and social impairment could be good candidates for *SHANK3* DNA testing including deletions, duplications or point mutations (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4489957/).

1.3.7.2. Genetic counselling

A general practical guideline was introduced for providing genetic counselling to ASD patients and their family, especially parents.

If a couple has a non-syndromic ASD child, the recurrence rate will be 8.4-fold increase (Hansen et al., 2019).

A karyotype and Fragile X test should be indicated for all ASD patients.

Prenatal genetic testing with the expectation for ASD detecting is still far from reality as the current prenatal screening methods are not powerful enough.

Patients with the presence of dysmorphic features and neurological symptoms should be referred for array-based CGH analysis. Recently, array-CGH become a first-tier test for all ASD patients with or without dysmorphia.

There is no specific genetic screening for the Neurexin genes such as *NLG3* and *NLG4* as the patients with these gene mutations do not share any specific clinical features and the frequency of these mutations is quite low.

Patients with severe verbal and social deficits should be considered for *SHANK3* genetic testing. But keeping in mind that some *SHANK3* mutations have also been found in unaffected siblings of ASD probands (Moessner et al., 2007).

MECP2 DNA test should be considered for all girls with ASD and mental retardation even if they do not show full symptoms of Rett syndrome. For example, girls with autism carrying *MECP2* mutations may have normal head size, no history of regression and no stereotypies or seizures (Lintas and Persico, 2009).

All ASD children combined with extreme macrocephaly (⩾3 SD) should be tested with *PTEN* mutations as *PTEN* haploinsufficiency may also produce tumour susceptibility. If the patients have a *PTEN* mutation, an oncological monitoring programme should be introduced for them (Zahedi Abghari et al., 2019).

1.4. Treatments of autism spectrum disorder

There is currently no therapy to cure ASD, but several interventions have been suggested for treatment. These interventions may help to reduce the severity of symptoms, improve cognitive ability and potentially gain more daily skills. For example, behavioral interventions focused on development of

social communication skills are especially helpful at younger age as children naturally gain these skills at this time, and it reduces restricted interests and repetitive behaviors. Some medications help to relieve the signs and symptoms associated with autism: Selective Serotonin Reuptake Inhibitors (SSRI) to manage anxiety or depression, antipsychotics to decrease behavioural abnormalities, anticonvulsants to control seizures and methylphenidate to treat attention deficit hyperactive disorder. Multiple approaches including education and medications are usually recommended for ASD treatment strategies. The most suitable approaches depend on the patient's age, strengths, challenges and differences with early intensive intervention potentially gaining better improvements. However, even receiving fully interventions, some children still remain significantly impaired. Alternative treatments are intensively investigated with the aim of improving the outcome of ASD treatment. Recently, stem cell transplantation is new treatment showing impressive improvements for different neurologic conditions with several studies reporting a promising outcome for the use of stem cells in the management of ASD. Understanding the genetic patterns and treatment principle may help to improve the effect of treatment. This is called personalized therapeutic intervention.

1.4.1. Behavioral Interventions

Behavioural interventions can be separated into a selection for children and another for adults. High-intensity applied behaviour analysis (ABA) could help to improve cognitive functioning and language skills in young children. Early intensive behavioural intervention (EIBI) based on the principles of applied behaviour analysis is a well-established therapy for young patients. 20 to 40 hours per week is the estimated time for substantial benefit for core ASD symptoms, especially communication skills. While, social skills intervention, for example group administered training, seems to have more positive benefits on older children. Behavioural therapy techniques within a social learning framework is recommended to be applied for the management and support of children and young people with autism by using social learning. This therapy includes using video modelling, peer feedback, imitation, and

reinforcement to teach conventions of appropriate social interpersonal interaction. Observational studies provided evidence of improvement of social interaction after joining a social skills group (Genovese and Butler, 2020).

1.4.2. Medication Treatments in ASD

It is still a challenge to apply psychopharmacology in the treatment of ASD and commonly occurring comorbidities since there is so much variability in presentation. ASD patients also appear to have more side effects from psychopharmacological agents than those of the same age without ASD. This method however could be useful for reducing some specific symptoms but the potential benefits and the risk of medications should be carefully considered on a case by case basis. General medications are often commonly applied including stimulants, alpha-2 agonists, antipsychotics, anticonvulsants, and antidepressants.

Currently, there are no medications that are officially approved for the treatment of ASD core symptoms such as social communication deficits or repetitive behaviours. Medications with evidence-based studies commonly target other symptoms including hyperactivity, inattention, impulsivity, irritability, aggression, self-injurious behaviour, repetitive behaviours (including stereotypies), and insomnia. The antipsychotics risperidone and aripiprazole are approved for treatment of irritability associated with ASD. Drug-drug interactions and their concentrations should be considered for any management of medication levels for each patient. In addition, different individuals may have a different metabolism based on their microsomal P450 enzyme system genotype patterns. Therefore, they may show different response to particular medications which could then put them at risk as either a failure of drug therapy and/or adverse side effects. Understanding of the metabolic differences between individuals also identifies the suitable drug dosage and therapeutic agents that may be prescribed- this is termed personalized medicine. Further development of resources for clinicians including pharmacogenetic dosing guidelines for medications are required for a better outcome of the treatment. Applying the

knowledge of the correlation between genetics and pharmacogenomics involving drug metabolism will benefit the treatment of many other psychiatric and behavioural problems. Research for new drugs or new purposes of existing drugs for behavioural problems treatment is still in investigation with indications of promising results. Furthermore, brain imaging techniques such as functional MRI or PET-CT scan could help to identify regions of the brains which are affected by ASD potentially leading to development of new treatments for a specific brain regions.

1.4.3. Stem cell transplantation

The pathophysiology and mechanisms of autism have not been thoroughly clarified. Among many theories, neural hypoperfusion and immune dysregulations have been reported to be the two major physiological alterations and are correlated with the severity of autism symptoms (Ichim et al., 2007). Hematopoietic stem cells (HSCs) have been proved to promote blood perfusion to the ischemic brain area through angiogenesis in animal models (Peterson, 2004, Park et al., 2009). Preclinical models on rats and hippocampus have shown that human umbilical cord blood mononuclear cells (HUCB MNCs) can significantly improve the microenvironment of the aged hippocampus and rejuvenate the aged neural stem/progenitor cells, therefore suppressing inflammation (Bachstetter et al., 2008, Shahaduzzaman et al., 2013). Mesenchymal stem cells (MSCs) demonstrated immunoregulatory properties by suppressing T cells, B cells, NK cells as well as dendritic cells (De Miguel et al., 2012, Shi et al., 2011).

1.5. Aims and objectives

Many studies carried out in Caucasian populations while ASD is not well studied in Asia with very limited information. The various prevalence of ASD reported from 0.09-1.07% in south Asia (Hossain et al., 2017), 0.1018% in China (Wang et al., 2018) and 2.64% Korea (Young Shin Kim et al., 2011). One report from 17,277 Vietnamese children from 18 to 30 months of age estimated the prevalence was 0.752% and the number is increasing (Hoang et al., 2019). The reasons for this various rate are still

unclear but some factors were suggested that influence the diagnosis of ASD including socioeconomic status, ethnicity and parental education level (Zaroff and Uhm, 2012, Mazurek et al., 2014, Thomas et al., 2011, Russell et al., 2011, Ka-Yuet Liu et al., 2010). Vietnam is a developing country with low medical, economic and education level, especially many countryside areas that may affect to the awareness and diagnosis level of autism. There are no published studies on the genetics of autism in Vietnam so this research will contribute to the ASD database and help shed light on whether genetic variants linked to ASD varies between populations. Identification of any genes related to autism spectrum may also be important for diagnosis, prognosis, genetic counseling and prenatal diagnosis for families with autistic children. Therefore, it is necessary to build up the typical genomic data of autism spectrum disorder of Vietnamese patients so that it can be used as a basis for developing diagnostic procedures for autistic children and pre-natal screening for families in need. Investigations primarily focused on laboratory analysis of chromosome structural variations and the associated implications on biochemical or developmental pathways. The primary objectives for this study are:

(1) Clinical characterization of ASD patients in Vietnam

(2) Identification of *de novo* CNVs in the recruited families and validation of the data

(3) Estimation of the CNV based genetic correlation of ASD for future genetic counselling in Vietnam

(4) Analyzation the candidate genes by pathway and network based analyses for further biological insights

CHAPTER 2: MATERIALS AND METHODS

2.1. Autism Project in Vinmec

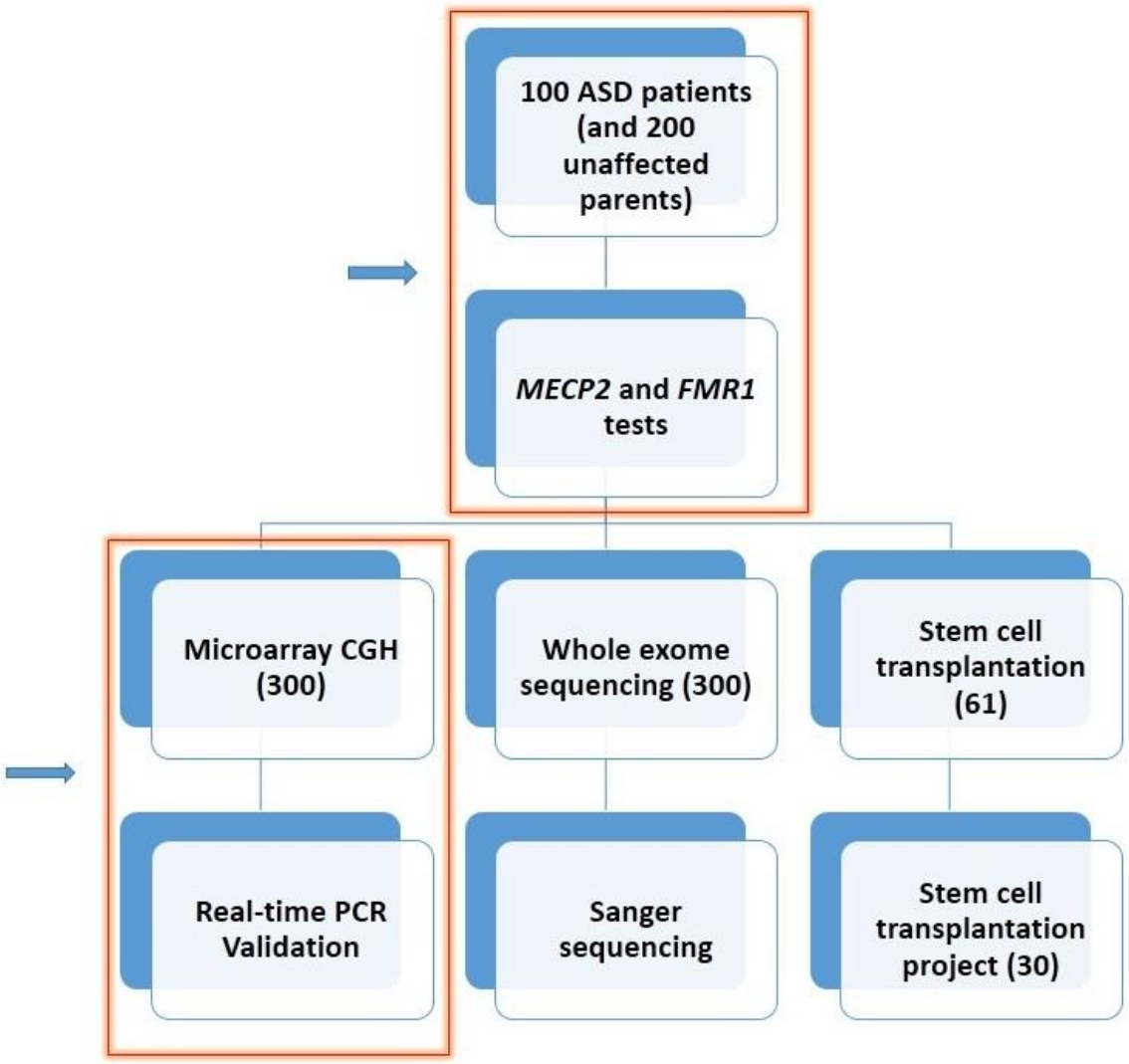

Figure 10: The ASD project in Vinmec

Orange boxes are parts related to my PhD thesis and my involvement at Vinmec. I took part in collecting 300 blood samples from 100 autism patients and their parents, 100 clinical dataset and follow up patients' improvement within 3 years of my journey. I also carried out almost laboratory experiments including aCGH and Real-time PCR.

Vinmec healthcare system had initiated a large project in where 100 ASD patients (and their unaffected parents) were recruited for genetic tests and assessments of treatment strategies (combination between

stem cell transplantation and education intervention) (figure 10). The research presented in this PhD dissertation was a key part of this larger project. Patient recruitment, collection of the clinical data for all 100 patients, microarray experiments and data analysis, real-time PCR validation and pathway analysis were the components, which are included in this dissertation.

2.2. Sample collection and clinical characterization

Children in Vinmec international hospital, Vietnam diagnosed with Autism Spectrum Disorder by Diagnostic and Statistical Manual of Mental Disorders (DSM), Autism Diagnostic Observation Schedule (ADOS) and/or Childhood Autism Rating Scale (CARS) were recruited to the research (details of each of the tools are described in the Introduction of this thesis and evaluation forms are presented in appendix 2,3,4). All patients were diagnosed based on three of the above criteria by physicians trained for ASD diagnosis at Vinmec International Hospital. The final classification of the disease followed the International Statistical Classification of Diseases and Related Health Problems 10^{th} Revision (ICD-10) guideline. The parents/carers of patients who met all criteria were invited to participate in the research. If they agreed, an interview was arranged at Vinmec International Hospital. During the interview parents answered some questions about their child/grandchild's health condition including family history, history of pregnancy, newborn's process of development and psycho-motor development, result of other tests, etc. The interview could last 10 to 15 minutes. Researchers collected information related to the patient's health from medical records (see Appendix 1).

This research was carried out in accordance with the World Medical Association's Declaration of Helsinki. This study is approved by the ethical review board for biomedical research in Vinmec International General Hospital JSC, Vietnam and Ethics committee of University of Salford, Manchester, United Kingdom. Following a description of the study, informed consent was obtained from the guardian of each patient. All sample collection, processing and storage of DNA samples and data is carried out at Vinmec.

2.3. DNA isolation and qualification

100 trios (autistic children and their unaffected parents) were recruited in this research. All families are unrelated. 3-4 ml of blood collected in EDTA-tubes was taken from the patients and their parents for molecular tests and genetic analysis.

QIAamp Blood Kit (Qiagen, Hilden, Germany) was used for genomic DNA extraction.

The quantity of DNA in all samples was measured by Nanodrop spectrophotometer. All samples had an A_{260}/A_{280} ratio more than or equal to 1.8 and concentration of DNA was 20ug/ml.

Agarose gel electrophoresis was used for randomly checking the quality of DNA in 10% of samples. Genomic DNA was run in 0.8% agarose gel prepared with 1X TBE buffer solution and 5% Red Safe on 80V for 30 minutes. After electrophoresis, the gel was visualized by the Versa Doc imaging machine with Image Lab 5.0 software.

2.4. Fragile X and *MECP2* DNA test

All patients had a Fragile X DNA test by examining the size of the trinucleotide repeat segment and the methylation status of the whole *FMR1* gene.

All female patients were indicated for a *MECP2* DNA test which was performed by running Sanger sequencing on exons 2, 3 and 4 of the *MECP2* gene.

2.5. Microarray-based Comparative Genomic Hybridization

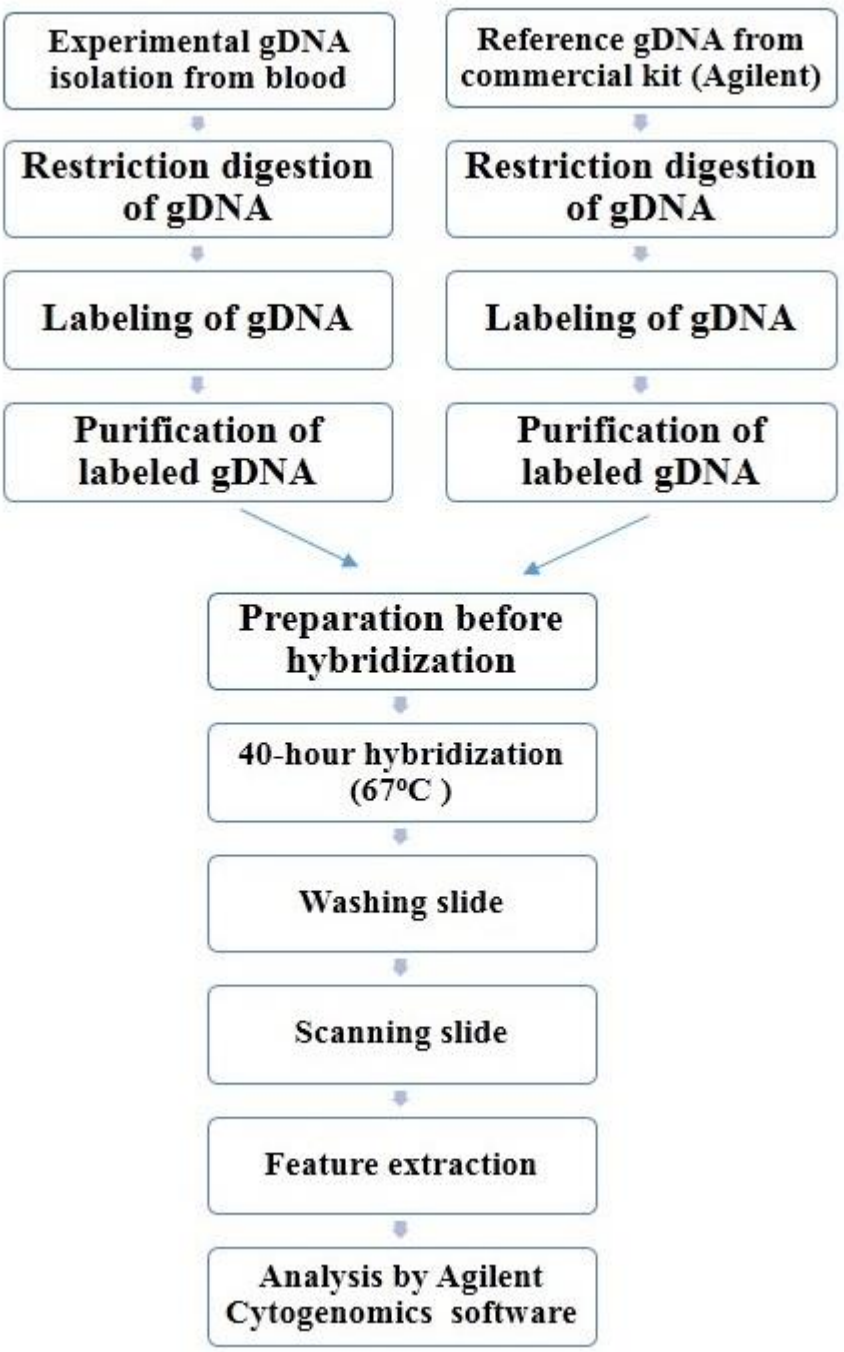

Figure 11: Direct workflow for sample preparation and microarray processing as recommended by Agilent.

We performed aCGH experiments on GenetiSure Cancer Research CGH+SNP Microarray, 2 X 400K KIT (Agilent) to detect CNVs. The experimental and analysis protocol was as advised by the manufacturer. The Agilent aCGH application uses a "two-color" process to measure DNA copy number changes in an experimental sample relative to a reference sample. The kit uses random primers and the exo-Klenow fragment to differentially label genomic DNA (gDNA) samples with fluorescent-labelled nucleotides. After purification of labelled gDNA, a DNA check was performed on Nanodrop with the quality Yield (µg): 8 – 13 and Specific Activity of Cyanine 3 and Cyanine 5 Labelled Sample (pmol/ µg): 20 – 60. Each specimen sample was mixed together with the corresponding reference sample. Then, samples were hybridized to Agilent slides at 67^{o}C for 40 hours. After washing, the slide was scanned by an Agilent Microarray Scanner. Agilent Feature Extraction Software 11.5.1.1 was used to assess the quality of image data file (.tif). And, the first-pass quality control (QC) filter of <0.25 derivative log-ratio threshold was applied.

2.5.1. Copy number variations calling and annotation

We used ADM-2 algorithm to call CNVs with following criteria: (1) up to 6.0 standard deviations, (2) three consecutive probes, (3) the minimum absolute log ratio is 0.25 as the default in ADM-2, (4) Fuzzy Zero = ON, and (5) Diploid Peak Centralization: ON. We also removed regions of called CNVs that belong to gain/loss regions in the Agilent reference. A loss/gain region in the reference will result in an apparent gain/loss in the sample.

A list of candidate genes for further analysis was sorted by using the following: (1) has at least one exon overlapped with called CNVs, (2) does not belong to the DGV stringent map (genes belong to the map are considered as benign) and (3) does not belong to any CNVs with frequency ≥1% in the NSTD100 database. It is possible that CNVs with frequency >1% may contribute as weak risk factors but such events are typically smaller and have not been sufficiently assayed by microarrays (Coe et al., 2014).

Candidate genes are then annotated with the following information: (1) The number of exons overlapped with called CNVs, (2) Phenotype and inheritance model obtained from the Clinical Genome Database, (3) DGV stringent map (Zarrei, Macdonald, Merico, Scherer, & Mg, 2015), (4) DGV gold standard CNV database (MacDonald, Ziman, Yuen, Feuk, & Scherer, 2014), (5) Pathogenic regions in the ISCA database (NSTD45), (6) 55 pathogenic regions in the NSTD100, and (7) Information obtained from SFARI and DECIPHER databases.

2.5.2. Copy number variations interpretation

- CNVs are classified into five main categories including pathogenic, likely pathogenic, variant of uncertain significance, likely benign and benign following the American College of Medical Genetics and Genomics (ACMG) guideline.
- A CNV is annotated as pathogenic if it covers known pathogenic regions in either ISCA or NSTD100 databases.
- CNV in a child is classified as inherited if it is one in 3 cases: same start and end point with a CNV in the parent(s), inside a CNV in parent(s), containing exactly same gene(s) in a CNV in the parent(s).
- If the *de novo* CNV consists of a candidate gene annotated as high likely pathogenic gene, it is considered as high likely *de novo* pathogenic CNV. Other CNVs are called *de novo* CNVs.
- Unique CNVs in the patients mean that they only present in the patients, not in 200 parents.
- Each CNV is described with full details including cytogenetic location, CNV size and linear coordinates with the genome build specified, state (gain or loss), genes containing.

2.6. Validation of CNVs

Quantitative real-time PCR (qPCR) was performed to validate CNVs. We chose *VPS29* gene as the endogenous gene with the primers designed on a location with no CNV detected in the UCSC genome browser database plus one candidate gene inside the CNV for validation. Primers were designed and

qPCR using SYBR green PCR Kit (Qiagen, Germany) was performed on a 7500 Real-time PCR System (Applied Biosystems, MA). Primers sequence are listed in the supplementary table 5. The results were interpreted by the 7500 Software v2.0.6. A higher or lower copy-number compared to input DNA will result in an earlier or later increase of fluorescence and was considered a signature for a deletion or a duplication. Quantification of target sequence was normalized and relative copy number (RCN) determined based on comparative ΔΔCt method with a normal control DNA as the calibrator. The ΔΔCt was calculated as follows:

ΔΔCt = (ΔCt unknown sample-ΔCt control sample). Normalized copy number = $2^{-\Delta\Delta Ct}$ A 0.5-fold RCN is used for deletion and 1.5-fold for duplication (Hussein et al., 2016).

2.7. Network analysis

For each ASD patient, we identified genes from their unique CNVs. Finally, a total of 958 genes were collected as candidates for ASD from all unique CNVs. We use enrichment analysis tools for finding the most likely candidate genes for further research on gene expression, and most related biological pathways. To find the most likely candidate genes, we used a candidate gene prioritization tool in ToppGene (Chen et al., 2009). The tool prioritizes the candidate genes based on functional similarity to a training gene list. The training list which are genes well-known to be associated with ASD present in our patients (including *SHANK2, SHANK3, CNTN6, CHKB, MAPK12, SBF1, LRP2BP, GABRB3*) (Abrahams et al., 2013). Training gene set were pasted in traning gene set box in the website and 958 test genes were pasted in test gene set box, and then submit. The candidate genes were ranked, then top ranked genes were listed and saved for further analysis in the future. To in-silico demonstrate associations between the promising genes and ASD, we used g:profiler (Reimand et al., 2007). The promising gene set was enriched with gene ontology (GO) terms and pathways. More specifically, the sub-categories of molecular function (MF), cellular component (CC) and biological process (BP) were used. In addition, pathways from KEGG (Kanehisa et al., 2010), Reactome (Fabregat et al., 2018) and

WikiPathways (Slenter et al., 2018) were used to enrich for the gene set. 958 genes were put into input gene box, then Gene Ontology terms were tick for choosing and then run analysis. Pathways with adjusted P value smaller than 0.05 were reported.

CHAPTER 3: CLINICAL CHARACTERISTICS OF STUDY PARTICIPANTS

3.1. Results

One hundred autistic children and their unaffected parents were recruited for the study. Of these families there were two families with two affected siblings and both of them are dizygotic twins. The rest of the patients were unrelated, as determined by their reported family history. Four patients had an uncertainty about their paternal origin (ASD080, ASD091, ASD100 and ASD101), identified by exome sequencing (Tran et al., 2020). Of the 100 patients in our study, 83 were male and 17 were female (male to female ratio ~ 5:1). All patients met at least one criteria of autism using the diagnosis tools (DSM, ADOS and CARS). The DSM results show that 79/97 patients were classified as level 3 – "requiring very substantial support" while CARS tool helped to group 95/98 patients in severe autism category with scores that ranged from 35 to 55.5 (average score of 46.8 points). The details are provided in table 3 and supplementary table 1 and 3. All patients showed the core symptoms of ASD: social-communication impairment and rigid restricted interests. Some patients also exhibited comorbid conditions such as hyperactivity (n=75), self-injurious behaviour (n=15), language regression during their early life (n=23), gastrointestinal (GI) problems (n=6) mostly chronic constipation and diarrhoea. Two cases showed congenital foot defects (ASD025 and ASD082). Secondary diagnosis were also recorded with ID (ten probands), EP (four probands), and cerebral palsy (CP) (one proband). Notably, six subjects had siblings with ASD (ASD004, ASD027) or with other neurological conditions, such as speech delay, CP and ID (ASD062, ASD065, ASD072 and ASD075). Of these, proband ASD004 and his male sibling were twins conceived through in vitro fertilization (IVF), while proband ASD027 and his sibling were natural pregnancies. All details are provided in supplementary table 1. The patients and family history were also recorded to identify any other neurodevelopmental or genetic disorders. Four families have at least one other member within three generations affected with autism and 19 others were found to be related to other individuals with neurodevelopmental disorders (table 4).

Table 4: clinical features of 100 probands: the diagnostic criteria and family history

Domain	**Male**	**Female**
Median age (years)	10.5 (range: 3-18 years old)	
Gender	83	17
CARS (follow the diagnosis from clinical psychologists)		
Severe (≥37)	81	14
Mild-Moderate (30-36)	2	3
DSM (follow the diagnosis from clinical psychologists)		
Level 3	70	12
Level 1-2	13	5
Secondary diagnosis		
Intellectual disability (ID)	7	2
Epilepsy	2	2
Cerebral palsy	1	0
None	73	13
Family history (up to 3rd cousin, excludes parents)		
None (sporadic)	64	13
Autism	4	0
Other neurodevelopmental disorders (for example ID, Epilepsy, Cerebral palsy, Parkinson and etc)	15	4

Imaging examinations which included brain magnetic resonance imaging (MRI), brain PET-CT were collected from medical record only in some patients. Forty (40) patients were indicated for brain MRI

by physicians and 15 showed abnormal signal changes including myelin degeneration, delay myelination, leukoaraiosis, ventriculomegaly and cerebellar atrophy. The remaining patients showed either no detectable abnormality or further information was not available (supplementary table 2). Twenty nine (29 out of 100) patients underwent dedicated 18F-fluorodeoxyglucose (18F-FDG) brain positron emission tomography-computed tomography (PET/CT). On post hoc analysis, the decreased and increased FDG metabolism regions in autistic children was evaluated based on a normal distribution curve. If the measured value was lower than a standard deviation (SD) from the median value of the standardized uptake values (SUV), it was considered to be hypometabolic while a measured value greater than a SD from the median value was considered to be an increase in metabolism (Sharma et al., 2018a). Evaluation of PET/CT images: Dark blue brain areas were defined as severe reduction in FDG metabolic rate, light blue brain areas were assessed as moderate metabolism while green was considered to have a mild metabolic reduction. The yellow-orange brain region was assessed as an increased FDG metabolism (Sharma, 2016). All of the PET-CT images in 29 patients showed hypometabolism in the brain with nine patients identified as a severe level of hypometabolism, 16 patients were moderate and four patients were mild level, respectively. While a patient could have damage in any one or even several parts of the brain, almost all of them showed hypometabolism in temporal lobe, parietal lobe, frontal lobe, hippocampus and limbic cortex in both sides of the brain. An example of PET-CT is in supplementary figure 1 and figure 2.

3.2. Discussion

In this project, we describe the differences related to morphological stratification of ASD probands based on clinical examination of ASD children that may help identify differences in ASD populations and further the possibility of identifying underlying causes. All patients were diagnosed with three different tools (DSM-V, CARS and ADOS) and they were chosen from both moderate and severe

groups (CARS from 35.5 point, DSM-V level from 2) – better level of the diagnostic accuracy and higher level of severity.

Our study reported that the male to female ratio is nearly 5:1. In general, observations across populations, including Vietnamese populations, indicate that the ASD-affected male/female (M/F) ratio is approximately 4-fold (Werling and Geschwind, 2013, Loomes et al., 2017, Hoang et al., 2019). There is a little different as the small sample size.

The earliest age at enrolment time is three years old because final diagnosis is made by age three, so we would like to have a proper diagnosis for our study participants. Even prior to that age, the symptoms of social-communication impairment starts may begin appearing but the characteristic behaviours and restrictions may not clear enough for proper diagnosis of autism. Although ASD is generally not be confirmed before three years of age, some signs could present from as early as one year of age. Early screening can be performed at 18 months and during the preschool years. But in some cases, early screening may miss detection of autism since many of the probands have a regression stage after a period of normal development. Early diagnosis and assessment are important for early intervention and to increase the potential for a good outcome (Zwaigenbaum et al., 2015). A family history of ASD should also raise a higher level of clinical concern. In our 100 patients, 77 patients were suspected before 36 months of age and the average age of diagnosis was 25.57 months. These mean that the early recognition of ASD in Vietnamese medical system is robust.

Currently, we cannot diagnose children affected ASD at a pre-symptomatic stage. Although no single cause can explain fully about ASD, studies provide evidence about the presence of abnormalities in brain structure or function in autism patients. We also know that the risk in other family members will increase with history of autism and the result from a recent large prospective study has shown that the recurrence rate was 18% in infant siblings and 33% in multiplex families. Early detection of ASD is very important because the connections in a baby's brain are most adaptable in the first three years of

life and the therapy applied has a greater impact to the improvement of skills and development (CDC, 2020). The treatment program set up before two years of age can make a big difference in both IQ and language, social skills. This helps ASD children can perform better in the later period of life. The sooner we can give the diagnosis, the earlier long-term treatment plan can provide to the patients that could afford these children and families the opportunity gaining the best outcomes.

It is important to consider the recurrence rate of autism among siblings in a family. Understanding any recurrence rate can aid in the interpretation of the genetic and environmental factors that may increase the familial risk for ASD which in turn could improve the quality of screening and counselling for the autism patients and their family. Palmer et al., reported in a study of 3,166,542 autism children that when the older sibling with ASD was a boy, ASD was diagnosed in 4.2% of female siblings and 12.9% of male siblings. When the older autistic sibling was a girl, ASD was diagnosed in 7.6% of female siblings and 16.7% of male siblings (Palmer et al., 2017). Out of 100 families in our study there were two families with two affected siblings (dizygotic twins) and two families with another affected relative. It was commonly found that there are minor problems with social communication skills or certain behavioural impairments in parents or relatives of the patients who have autism.

CHAPTER 4: GENETIC AND GENOMIC ANALYSIS OF STUDY PARTICIPANTS

4.1. Results

We screened all 17 ASD females for mutations in exons 2, 3 and 4 of the *MECP2* gene for Rett syndrome exclusion and test all 100 patients for *FMR1* gene. None of the 100 patients were positive for RETT syndrome and Fragile-X syndrome and thus no samples were excluded from aCGH.

And then, we performed aCGH experiments on all 300 participants (100 ASD patients and their parents) and analysed 12 million data points to score CNVs.

The quality of the experimental samples was checked in four steps: the first step was checking input DNA quality in gel agarose electrophoresis: the second step was checking of restricted DNA quality in agarose gel electrophoresis; the third step was checking the quality of labelling products by Nanodrop; and the final step was checking the quality of aCGH data by the Cytogenomics software (provided by Agilent) after experiments were performed.

All 300 samples passed the QC with high quality in control metrics of the cytogenetics software.

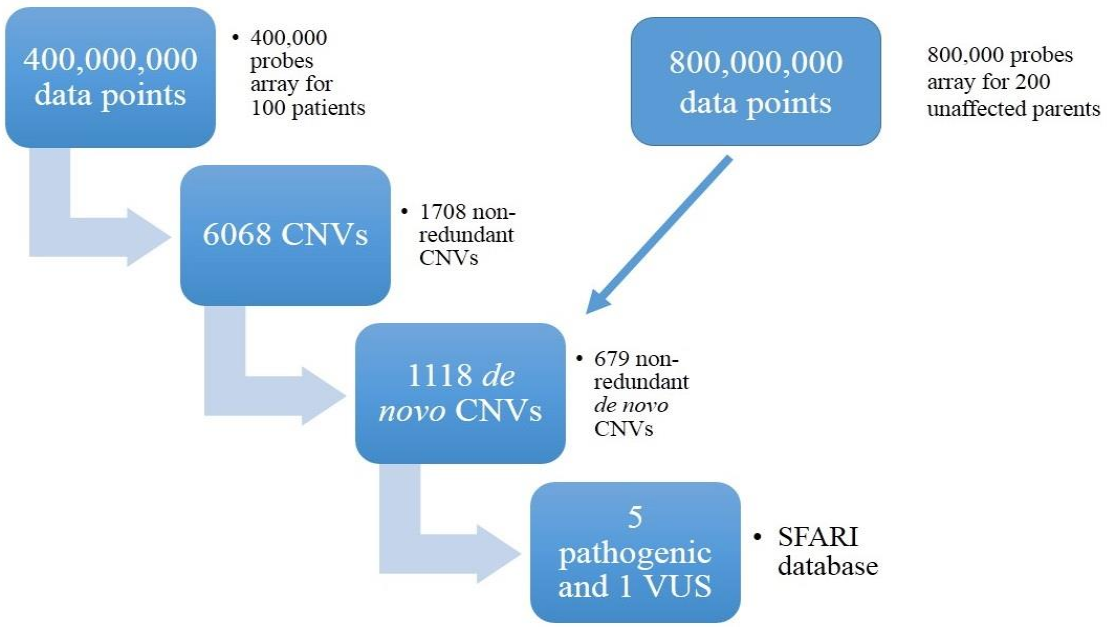

Figure 12: Data summary of *de novo* copy number variations (CNVs) in 100 ASD patients

A total of 12 million data points were analyzed. 6068 CNVs were found in 100 patients of which 1118 were de novo after removing common CNVs present in either parent. Out of all de novo CNVs, 679 were non-redundant. Based on the biggest autism database (SFARI) and the guideline from American College of Medical Genetics and Genomics (ACMG), five CNVs out of 679 were classified as pathogenic and one as variant of uncertain significance (VUS) related to autism by filtering via SFARI database.

4.1.1. CNVs in 100 ASD autism, contribution of inherited and *de novo* CNVs

In total 12,000,000,000 data points were analysed in the 100 patients and their unaffected parents. We observed 6068 CNVs (1708 distinct/non-redundant CNVs) of which 1118 CNVs were *de novo* (679 distinct CNVs). In 679 non-redundant *de novo* CNVs, there are 285 unique CNVs (CNVs present uniquely in patients, not in 200 normal parents) with 118 CNVs containing at least one gene. We reported five pathogenic CNVs from SFARI database (the strongest ASD evidence database) as the candidates for our interpretations (Figure 14) and we also reported 1 other VUS CNV that were confirmed by Realtime PCR. The contribution of deletion and duplication CNVs in each category of CNVs including all non-redundant CNVs in 100 patients, *de novo* CNVs and unique CNVs in each chromosome is shown in figure 15 and figure 16.

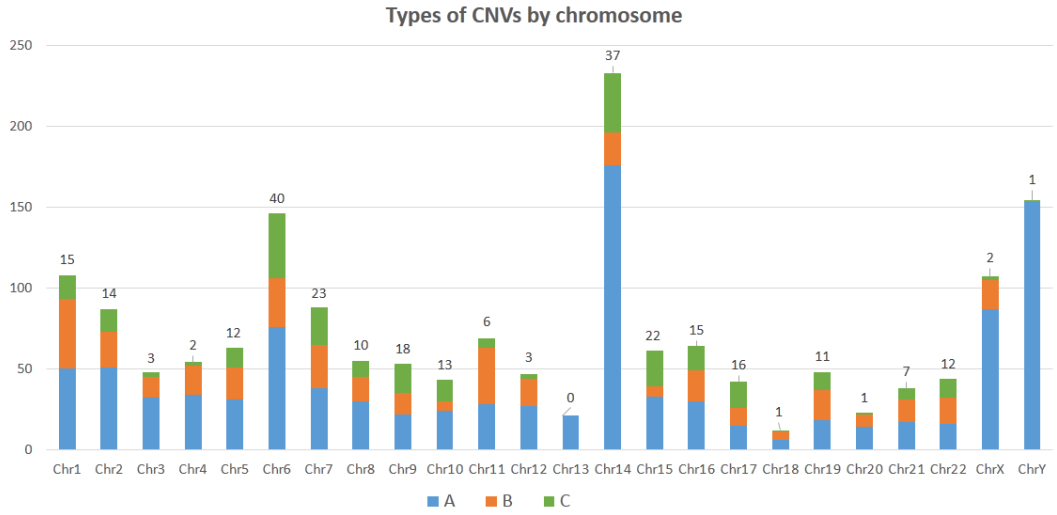

Figure 13: Distribution of unique, de novo and all non-redundant CNVs across the genome for each chromosome overlapping with SFARI

C: unique CNVs (with the number on top of green bar for each chromosome)

C+B: De novo non-redundant CNVs

C+B+A: All non-redundant CNVs

It is interesting to note that for chromosome 13 we don't find any de novo candidate CNV and only one de novo CNV has been found in total 154 CNVs on chromosome Y.

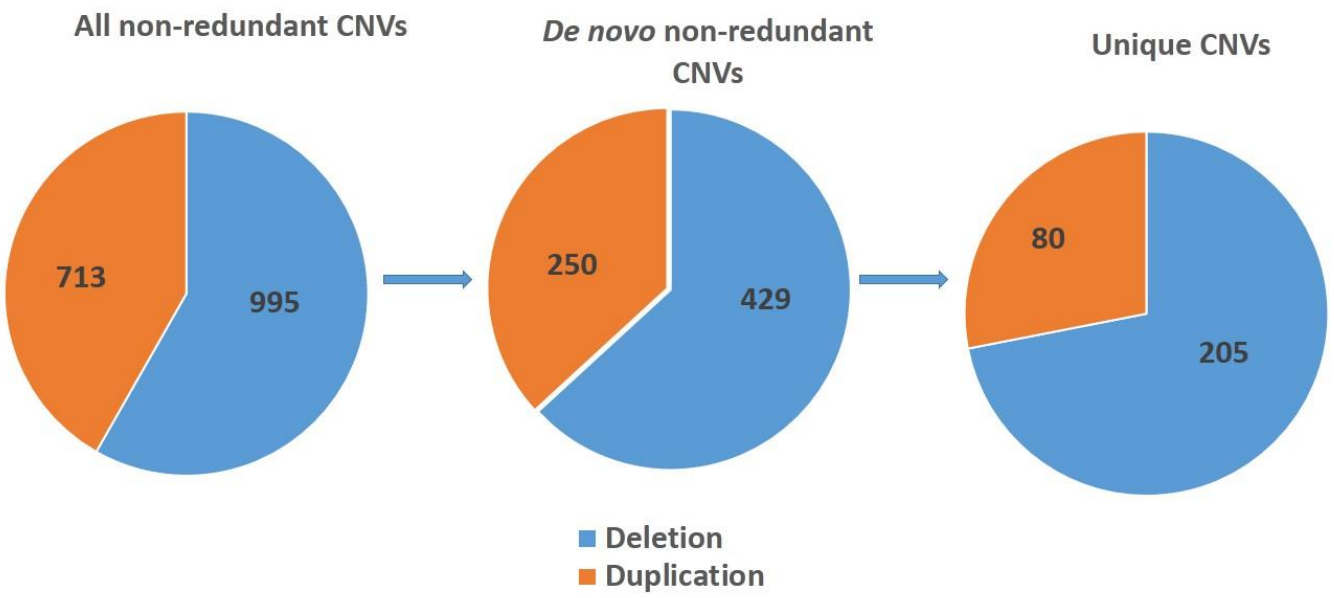

Figure 14: Contribution of deletion and duplication CNVs in each category of CNVs

In all non-redundant CNVs, a quite similar contribution of deletion and duplication was seen. The deletion CNVs are nearly double the number of duplication CNVs in the de novo non-redundant category. In unique CNVs, the number reduced to three.

We identified six *de novo* CNVs in SFARI database in seven different patients (three females and four males) and among these CNVs, there were two duplication CNVs and four deletion CNVs.

All identified *de novo* CNVs were classified according to the ACMG Guidelines (South et al., 2013). Five pathogenic CNVs were found in six patients and one VUS CNV in one patient. A summary table of all CNVs is shown in the Table 5. Table 6 contains details of the clinical and molecular details of candidate genes. In addition, there are unique 118 CNVs containing at least one gene are listed at supplementary table 4.

Table 5: Details of *de novo* CNVs in SFARI for aCGH test in 100 trios

Patients ID and gender	Chromosome region	Start-End point	Size (bp)	Aberration type	Representative gene(s) within the CNV	Clinical diagnosis	Classification
ASD025.1 (F)	11q13.2-q13.4	67,856,136-73,062,386	5,206,250	Loss	*SHANK2* (23/23 exons), *DHCR7* (9/9 exons), *FGF*	ASD/ID	Pathogenic - Autism
ASD047.1 (M)	22q13.32-q13.33	48,746,241-51,178,264	2,432,023	Loss	*SHANK3* (25/25 exons), *CHKB* (11/11 exons), *MAPK12* (12/12 exons), *SBF1* (41/41 exons)	ASD/ID	Pathogenic - Phelan-McDermid syndrome deletion
ASD080.1 (F)	22q13.33	51,137,326-51,170,223	32,897	Loss	*SHANK3* (9/25 exons)	ASD	Pathogenic -

							Phelan-McDermid syndrome
ASD089.1 (M)	22q13.33	49,564,639-51,178,264	1,613,625	Loss	*SHANK3* (25/25 exons), *CHKB* (11/11 exons), *MAPK12* (12/12 exons), *SBF1* (41/41 exons)	ASD/ID	Pathogenic - Autism Phelan-McDermid syndrome deletion
ASD091.1 (F)	4q34.1-q35.2	175,897,368-190,896,674	14,999,306	Loss	*LRP2BP* (9/9 exons)	ASD	Pathogenic Autism
ASD097.1 (M)	15q11.1-q13.3	20,102,541-32,445,252	12,342,711	Gain	*GABRB3*	ASD/ID	Pathogenic - 15q13.3 duplication
ASD088.1 (M)	3p26.3	1,414,674-1,435,453	20,779	Gain	*CNTN6* (6/23 exons)	ASD	Autism
ASD083.1 (F)	19q13.42	54,625,063-54,650,201	25,138	Gain	*PRPF31*, *CNOT3*	ASD	VUS

Table 6: Clinical and molecular details of the candidate genes (references SFARI and OMIM)

GENE	OMIM	Gene function	SFARI	Associated neurological disorders
SHANK3	1.Phelan-McDermid syndrome (606232) 2.Schizophrenia 15 (613950)	Shank proteins are multi-domain scaffold proteins of the postsynaptic density that connect neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein-coupled signalling pathways. Shank proteins also play a role in synapse formation and dendritic spine maturation	Score 1S	DD/NDD, ASD, ID, EPS
SHANK2	Autism susceptibility 17 (613436)	Shank proteins contain multiple domains for protein-protein interactions and function as molecular scaffolds in the postsynaptic density (PSD)	Score 2	ASD, EPS, ID, EP
GABRB3	Epileptic encephalopathy, early infantile (617113)	The encoded protein is a subunit of GABA-A receptor. Neurotransmission is predominantly mediated by a gated chloride channel activity intrinsic to the receptor	Score 2	ADHD, ASD, ID, DD/NDD

22q13 deletion (*SHANK3* gene)

Three patients (ASD047.1, ASD080.1 and ASD089.1) have deletion CNVs containing *SHANK3* gene and defined as pathogenic for ASD (Scored 1s in SFARI gene scoring). Details of CNV position are described in supplemental figure 2. These CNVs were absent in all 200 unaffected parents – implicating its pathogenic role. Below we describe identified patients and their CNVs.

Patient ASD047.1 is an 8-year-old boy born from a normal pregnancy and delivery history, diagnosed with autism (ICD-10: F84.0 – DSM 5 level 3, ADOS 22/12 and CARS 49.5) and Intellectual Disability (ID) (ICD-10: F70). His great aunt was also diagnosed with ID. He went to Paediatric National Hospital at 8 months with ID signs. He currently has poor communication skills defined by poor eye contact, expressionless face, lack of expressive speech and limit in daily self-care. He also has a disrupted sleep. He was found to have a *de novo* 2.43 Mb deletion CNV which contains *SHANK3* gene.

Patient ASD080.1 is a 5-year-old girl diagnosed with autism (ICD-10: F84.0 – DSM 5 level 3, ADOS 21/12 and CARS 49.5) with normal family history. Her mother had normal pregnancy and she was born at 40 weeks of gestation without any complications. The proband showed significant psychomotor development delay from birth but she was able to walk at two years of age. Currently, she could only use few single words, has limited social interaction such as plays alone, no response with her name and lack of eye contact. She has hyperactivity and numerous repetitive actions. She has a trouble with sleep. PET-CT image demonstrates hypometabolism in frontal lobe, parietal lobe, hippocampus and limbic cortex. The array CGH test found a 32 kb deletion which encompasses 9/25 exons of *SHANK3* gene in this patient. This patient was reported as having an unclear biological relationship with her father and this CNV was not found in her biological mother.

The patient ASD089.1 is a 5-year-old boy diagnosed with (ICD-10: F84.0 – DSM 5 level 3, ADOS 20/12 and CARS 54) and Intellectual Disability (ID) with normal maternal pregnancy and family

history. He had asphyxiation after birth (no Apgar score information). He can walk unsupported at two years of age. He lacks expressive speech, has limited social communication and could not do basic self-care. He has hyperactivity, restricted interest such as turning on-off the light, open-close doors. He has a decrease perception of pain. He has a 1.61 Mb deletion containing the entire *SHANK3* genes.

4q34.1-q35.2 deletion

This *de novo* CNV was only present in patient ASD091.1 and absent in all 200 unaffected parents. Patient ASD091.1 is an 8-year-old girl diagnosed with autism (ICD-10: F84.0 – DSM 5 level 3, ADOS 22/12 and CARS 51) from a normal family history. Her mother had to take hormonal drugs for a risk of miscarriage at eight weeks of pregnancy and caesarean delivery due to early amniotomy at 36 weeks of gestation. She currently shows lack of verbal communication, eye contact, and other social interactions. She also has hyperactivity, self-harms and has restricted activity such as striking toys to her teeth, or to the floor, biting. She is unable to do basic self-care. The patient was found to have a 14 Mb deletion CNV at 4q34.1-q35.2. This individual has concern about biological relationship with her father, but the mother does not have this CNV.

11q13.2-q13.4 deletion (candidate gene *SHANK2*)

This *de novo* CNV was only detected in patient ASD025.1 among the total of 300 samples. Patient ASD025.1 is a 6-year-old girl diagnosed with autism (ICD-10: F84.0 – DSM 5 level 3, ADOS 17/12 and CARS 45.5) and ID (ICD-10: F70) with a normal family history. Her mother suffered from Rubella infection one month before pregnancy. The child was born with birth weight 2800g without any complications at 38 weeks. She had flat feet and showed psychomotor delay, for instance, walking at two years and babble at three years of age and reduced gross and fine motor skills. She now lacks speech as well as non-verbal communication. She also has repetitive activity like turning around the wheel and propellers. She had a 5 Mb *de novo* deletion CNV containing many genes and *SHANK2* is the most significant candidate for ASD.

15q11.1-q13.3 duplication (candidate gene *GABRB3*)

Only patient ASD097.1 had this CNV classified as *de novo*. It is not present in any other samples in this study (including all unaffected parents).

Patient ASD097.1 is a 13-year-old boy diagnosed with autism (ICD-10: F84.0 – DSM 5 level 3, ADOS 22/12 and CARS 52) and ID (ICD-10: F70). The mother had normal pregnancy, delivery and family history. Psychomotor development was significantly delayed from birth, but he was able to walk unsupported at two years of age. Currently, he presents with disorders of social interaction, lacks expressive speech and produces inarticulate sounds. He exhibits numerous repetitive behaviours and hyperactivity. The patient has a 12 Mb *de novo* duplication containing many genes that have been reported in ASD database and covers two pathogenic regions (15q13.3 duplication).

3p26.3 duplication (candidate gene *CNTN6*)

There were two patients with CNVs in this region. The patient ASD088.1 has this as a *de novo* CNV while ASD012.1 inherited it from his father. This was otherwise absent is all other patients and controls.

Patient ASD088.1 is a 3-year-old boy diagnosed with autism (ICD-10: F84.0 – DSM 5 level 2-3, ADOS 20/12 and CARS 39). He was born at 39 weeks and the birth weight 3600 gam. The proband could walk at 13 months of age and started to babble at nine months. Currently, he can use few words and prefers to use English, for example counting, colours, and only uses verbal communication when he is asked. He only recognizes close family members and limits contact with peers. The patient is hyperactive, attention-deficit and repetitive. He has sensory disorder such as liking thrilling games, has sensitivity to strong light and is sensitive with some sounds. The aCGH experiment showed a 20Kb duplication in 3p26.3 containing 6/23 exons of *CNTN6* gene.

Patient ASD012.1 is a 14-year-old boy diagnosed with autism (ICD-10: F84.0 – DSM 5 level 2, ADOS 20/12 and CARS 43) with a normal family history. He was born with C-section without any complications and the birth weight was 3200 gm. He started walking at 12 months of age but had

language delays. The diagnosis age was 18 months and he has been offered education therapy until now. He can use only a few single sentences and he still uses meaningless sounds. He can recognize family members and finds the way be back home if he is close. The proband still shows deficiency in social interactions and communication: he plays alone, does not deal with peers and avoids eye contact. He also has hyperactivity, repetitive actions and sensory disorder. The individual could do some daily self-care activities.

19q13.42 duplication (candidate gene *CNOT3*)

Patient ASD083.1 mentioned above also has a 25Kb *de novo* microduplication at chromosome 19q13.42. This CNV contains 10/17 exons of *CNOT3* gene related to Intellectual developmental disorder with speech delay, autism and dysmorphic facies (autosomal dominant inheritance). This CNV is classified as VUS CNV.

All pathogenic CNVs and 3p26.3 duplication were validated by Real-time PCR and passed the validation. The real-time PCR results is in the figure 17. While 19q13.42 microduplication was checked by real-time PCR (primers were design in *CNOT3* gene) and showed no abnormality in this position.

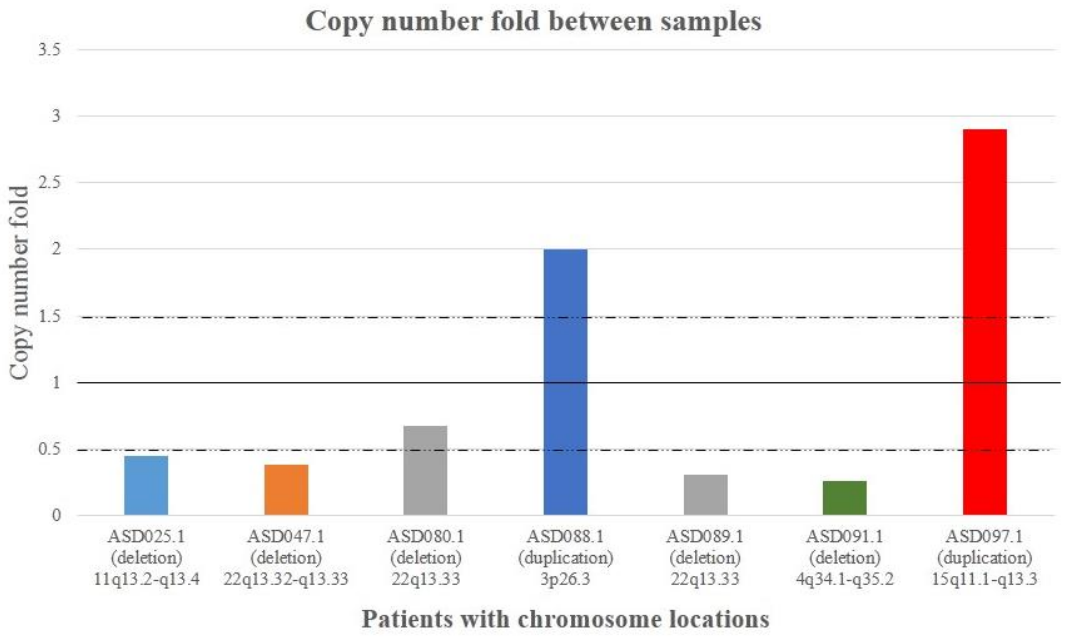

Figure 15: Representative Real-time PCR for aCGH data validation

The Real-time PCR experiment was applied to validate the aCGH results. Copy number fold shows the number of copies in each chromosome location with "1" means 2 copies in normal reference. The graph represents the validation of 4 deletion CNVs in 5 different patients and 2 duplication CNVs in 2 participants.

4.1.2. Candidate Gene Prioritization & Enrichment Analysis

The candidate genes were ranked (See supplementary file 1), then the top 10 ranked ones (*GABRA5, CHRNA7, CACNA1A, GABRA3, APBA2, GABRG3, MAPK8IP2, CACNA1H, CYFIP1, DRD5)* were selected as the most promising genes for analysis in the further research (details in table 7).

Table 7: Rank of the first 10 candidate genes and their parameters

No	Gene	Disease Score	Disease P Value	Average Score	Overall P value
1	*GABRA5*	0.986	1.94E-04	0.860	9.80E-08
2	*CHRNA7*	0.993	1.94E-04	0.737	5.98E-07
3	*CACNA1A*	1.000	1.94E-04	0.713	2.15E-06
4	*GABRA3*	0.606	0.009	0.661	3.96E-06
5	*APBA2*	0.463	0.012	0.733	7.44E-06
6	*GABRG3*	0.746	0.004	0.624	1.27E-05
7	*MAPK8IP2*	0.250	0.019	0.723	6.28E-05
8	*CACNA1H*	1.000	1.94E-04	0.625	1.48E-04
9	*CYFIP1*	0.463	0.012	0.660	1.60E-04
10	*DRD5*	0.4471	0.013	0.640	1.64E-04

Statistically significant enriched GO terms/pathways were selected if an adjusted P-value of a multiple-test correction was not larger than 0.05 (Reimand et al., 2007). The first 10 pathways with the highest

adjusted P value mainly related to synaptic function or neurotransmitter including chemical synaptic transmission, anterograde trans-synaptic signalling, trans-synaptic signalling. The figure 18 lists top 5 pathways with the smallest adjusted P value. This means that genes inside unique CNVs strongly relate to synaptic functions and may contribute to the development of ASD.

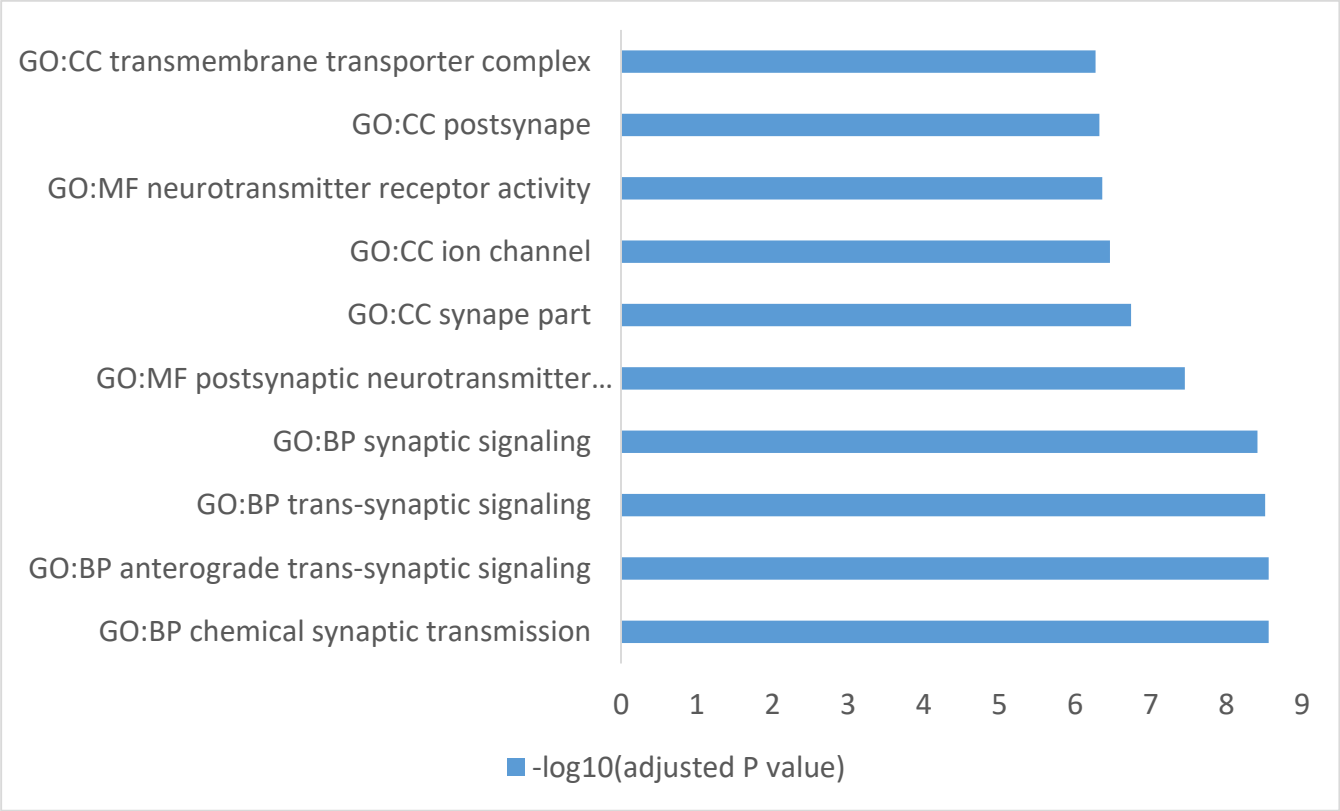

Figure 16: The enrichment analysis result (See supplementary figure 2 for more detail)

The promising gene sets were enriched with gene ontology terms and pathways with the smallest adjusted P value. The top 5 all relate to synaptic functions.

4.2. Discussion

This is the first ASD genomics study in a Vietnamese population. In this project, we focussed on potential utility of the aCGH based *de novo* CNV analysis for genetic diagnostic of autism. We found that in our study cohort 7% of ASD patients can be diagnosed based on CNVs in candidate genomic regions. This is in line with other recent reports where the diagnostic success was between 7%-9% (Shen et al., 2010, McGrew et al., 2012), confirming its potential clinical helpfulness as a first tier

genetic testing for ASD patients. A similar contribution of deletion and duplication CNVs were seen in the total CNVs but it changes significantly in *de novo* and unique CNVs. The proportion of deletion CNVs was much larger in term of unique CNVs compared to duplication CNVs. This may suggest a role for deletion CNVs in the patients or may just be a bias in patient selection.

Some CNVs containing very significant genes were seen more often with 3 different patients having a deletion involving the *SHANK3* gene. Shank/ProSAP proteins encoded by the three genes *SHANK1, SHANK2 and SHANK3* are master scaffold proteins, located at the post-synaptic density of glutamatergic synapses and are essential for synaptic development and functions. There is strong evidence supporting the association between those genes and ASD (Guilmatre et al., 2014). The Phelan-McDermid syndrome (PMS) was the first pathology report in patients with an alteration in *SHANK* genes (Phelan et al., 2001). This syndrome is described as a neurological deficit characterized by global developmental delay, moderate to severe intellectual impairment, absent or severely delayed speech and neonatal hypotonia. Over 50% of patients with this disorder have autism or autistic-like behaviour so it could be included in ASD (Shin et al., 2015). Phelan-McDermid syndrome is caused by deletion or changing in chromosomal structure in 22q13 region or mutations in *SHANK3* gene (Bonaglia et al., 2011). Based on this, three patients in our study (cases ASD047, ASD080 and ASD089) were diagnosed with Phelan-McDermid syndrome (MIM 606232) accounting for 3% of study population. The clinical features of PSM syndrome including developmental delay/intellectual disability, hypotonia, absent or delayed speech, self-stimulation behaviour seizures and decreased perception of pain and they may have physical manifestations such as cardiac, renal abnormalities and gastrointestinal dysfunction. But our three patients do not show all these features and there is no significant different with other ASD patients. The patient ASD080 has a smaller deletion containing a part of S*HANK3* gen while the two others have a larger deletion including whole *SHANK3* gene and few other genes. *SHANK3* gene encodes a scaffold protein in the postsynaptic densities of excitatory synapses which plays a role in the

connection of membrane-bound receptors to the actin cytoskeleton. Loss of one functional copy (haploinsufficiency) of *SHANK3* gene is known to have a causative role in a monogenic form of ASD with a frequency of 0.5% (Kolevzon et al., 2014). A higher frequency of CNVs involving *SHANK3* is reported in this project (3%) compared to the prevalence in Chinese cohort (1.7%), and 20 studies of European, American, and Australian populations (0.24%) (Gong et al., 2012, Shin et al., 2015), suggesting a possible higher frequency of Phelan-McDermid syndrome in DD/MR/ASD patients among East Asians (Bonaglia et al., 2011). A large number of *SHANK3* mutations recently have been found in many ASD patients without Phelan-McDermid syndrome that strongly implicates *SHANK3* deficiency by disruption or mutations as one of the most frequent and penetrant monogenic causes of ASD and socio-communicative impairments (Leblond et al., 2014). In recent research, mutations in *SHANK1* and *SHANK2* have also been involved with ASD (Leblond et al., 2014), supporting the idea that there is a general function for these proteins working within common molecular pathways associated with ASD (Jiang and Ehlers, 2013). In our study, we report an ASD case with a deletion in 11q13.2-13.4 region containing the *SHANK2* gene. Different independent studies have supported evidence for the involvement of *SHANK2* in ASD and ID (Berkel et al., 2010, Pinto et al., 2010, Wischmeijer et al., 2011, Leblond et al., 2012, Chilian et al., 2013, Schluth-Bolard et al., 2013). Berkel *et al.* 2010 reported two independent *de novo SHANK2* deletions in two individuals, one with ID and another one with ASD. In addition, a *de novo SHANK2* deletion was detected in a patient with ASD by using Illumina 1M single nucleotide polymorphism (SNP) arrays for performing whole genome analysis and a second patient was described by the AGP, 5237_3, is patient SK0217-003 reported in Berkel *et al.* 2010 (Berkel et al., 2010). Another had also reported that *de novo SHANK2* deletions appeared in patients with ASD and concluded that several *SHANK2* variants reduce the number of synapses *in vitro* (Leblond et al., 2012). Although evidence of the correlation between *SHANK* genes and ASD is increasing, *SHANK* mutations are believed to affect only a limited number of patients. Therefore,

sequencing of these genes is not considered as a routine test in clinical practice. In addition, sequence and annotation of these genes is not fully understood and could lead to incorrect interpretations. Since the clinical impact of *SHANK* gene mutations is still mainly unknown, more studies are needed. Despite these concerns, recent studies still suggested that *SHANK* mutations might be more frequent in patients with ASD than expected previously with some specific clinical profiles identified in those patients (as discuss in the introduction part). Thus, extended clinical investigation in ASD patients having *SHANK* mutations can be considered.

The 15q11-q13 duplication syndrome is characterized by autism, mental retardation, ataxia, seizures, developmental delays, and behavioural problems (Bundey et al., 1994, Burnside et al., 2011). A boy with an extensive mental retardation, infantile autism, ataxia, and seizures was identified with this deletion (Bundey et al., 1994). An earlier paper described a patient who possessed a less extensive duplication was reported by Clayton (Clayton-Smith et al., 1993). This patient had ataxia and moderate developmental delay, particularly of language, but neither epilepsy nor behaviour problems. Miller's group (Miller et al., 2009) reported five patients, including two siblings, with a chromosome 15q13.2-q13.3 microduplication detected by aCGH. Among the five candidates, there were four patients diagnosed with autism and the fifth had some repetitive behaviours and expressive language delay. No consistent pattern of dysmorphology or seizures were recorded. Three patients of these, including the two siblings, inherited duplication from an apparently unaffected mother. The duplications size ranged from 0.50 to 1.98 Mb. One report using microarrays identified 69 (0.41%) individuals in a total of 17,000 subjects with detected deletions and 77 (0.45%) with duplications of BP1-BP2 on proximal 15q. Among those patients, only 56 cases with deletions and 49 cases with duplications had available phenotypic information. These symptoms included autism, developmental delay, motor and language delays, and behavioural problems, consistent with a spectrum of neurologic impairment. It was concluded that copy number variation of break point 1-break point 2 (BP1-BP2) is not sufficient to

cause a phenotype, but may increase susceptibility to neurodevelopmental problems (Burnside et al., 2011).

A 15Mb deletion at chromosome 4q34.1-q35.2 is classified as a pathogenic CNV with many reported evidence (Bendavid et al., 2007, Caliebe et al., 1997, Cingoz et al., 2006, Descartes et al., 1996, Lin et al., 1988, Pickard et al., 2004, Van Buggenhout et al., 2004). Interstitial deletions at 4q34-q35 position usually do not cause major anomalies but few reported features such as mild to moderate mental retardation, congenital heart defects, facial dysmorphisms (Bendavid et al., 2007, Cingoz et al., 2006, Pickard et al., 2004). The severity of the phenotype depends on the size and position of deletion. 94% and 65% patients with 4q34-q35 deletion have developmental delay and cognitive/motor delay respectively (Cuturilo et al., 2011, Middlemiss, 2011). Yong et al (2017) reported a 12-year-old boy having a 1.2 Mb deletion at 4q35.2 with autism and intellectual disability diagnosis during childhood. He was born at 33 weeks of gestation with complications from maternal diabetes and alcohol use. He also showed other abnormalities including left cephalohematima, patent ductus arteriosus, atrial shunt (Youngs et al., 2012). Chien et al (2010) detected an 8-year-old boy who carries a 6.8Mb *de novo* deletion at chromosome 4q35.1-q35.2 with hypotonia in his infancy and autism at 1 year and 10 months. He showed no dysmorphic features and mental retardation (Chien et al., 2010). This CNV contains The fat tumour suppressor (Drosophila) homolog 1 (FAT1) gene involved in nerve cell adhesion and is part of the cadherin gene family, important in many developmental processes and autism (Dunne et al., 1995, Abou Jamra et al., 2008, Chien et al., 2010).

A 20 Kb microduplication at chromosome 3p26.3 contains 6/23 exons of *CNTN6* gene found in patient ASD088.1. *CNTN6* copy number variants had detected in 13 patients with various neurodevelopmental disorders including developmental delay, autism spectrum disorder, seizures and attention deficit hyperactivity disorder. Among them, five patients had a duplication of this gene. The size of CNVs ranged from 93Kb to 1.23 Mb (Hu et al., 2015). *CNTN6* gene encodes a member of the contactin family

and contribute to the generation of oligodendrocytes (Cui et al., 2004). Deficiency of this gene in mouse model showed the relation with impaired motor coordination and abnormal apical dendrite projections of deep layer pyramidal neurons in the visual cortex (Takeda et al., 2003, Ye et al., 2008). Furthermore, *CNTN6* gene also reported as a part of the heteromeric receptor complexes (Zuko et al., 2011). Therefore, CNVs containing this gene may cause the dysfunction of the receptor complex and affect brain and nervous system (Hu et al., 2015). Although, the deletion/duplication containing this gene is frequenly report in the control population (MacDonald et al., 2013). We also found another patient inherited a bigger duplication (578 Kb) containing 6/23 exons of *CNTN6* gene from his father (patient ASD012.1). Therefore, the genotype-phenotype correlation is still unclear, the CNV is classified as VUS.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE PROSPECTS

Better understanding of the role of genetics in ASD will improve diagnostic and treatment outcomes. Known causes ASD account for around 10% of all ASD cases, mostly from microscopically visible chromosomal abnormalities or clearly defined syndromes such as Fragile X and Rett syndrome. The application of high resolution techniques and novel sequencing technologies have the potential to increase the detection of single-gene and sub-microscopic genomic alterations including chromosomal rearrangements, microdeletions and microduplications. However, any novel rare variants found would be challenging in term of interpretation. In order to strengthen the confidence of results, larger numbers of patients and controls are required and detailed phenotypic evaluation of cases and controls will be necessary. In some cases, the variant is very rare and even large numbers of patients might not be enough for a confident finding. Early behavioural observation is part of standard of clinical screening for autism. Medical geneticists can combine clinical features and information with the appropriate cytogenetic and molecular genetic tests for evaluation of ASD patients. Identification of genetic subtypes would be important for the development of biomarkers for the useful evaluation of disease progression and effectiveness of treatments. Genetic panels for ASD may become a part of newborn screening in the near future since early diagnosis could improve treatment outcomes. In any family with a syndromic ASD proband, genetic counselling is very important to reduce the recurrence.

Our study shows that Vietnamese ASD patients have similar genomic features from a *de novo* CNV perspective and CNV screening can be used as a first-tier test diagnostic for clinical management. Also, we found the highest (3%) contribution of deletions involving the *SHANK3* gene, which could pave the way for future diagnostics focused on this gene alone by real-time PCR with few couple of primers with a cheaper price, especially in the Vietnamese population. This also helps to diagnose PSM syndrome in ASD population.

Limitations of our study include a relatively small sample size and missing of some clinical results. Not all of the patients had the brain imaging, which may have skewed our final morphological classification through a lack of quantitative evidence. Additionally, none of 100 patients underwent an IQ test which might be considered as an incomplete evaluation of the patients' clinical features. However, any gains in understanding the effects of clinical heterogeneity along with better diagnostic tools will help to improve the accuracy of diagnosis and then, gain more experience in clinical assessment, genetics, and treatment approaches in autism spectrum disorder.

Further NGS applications such as whole genome sequencing, epigenetics, transcriptomic, and proteomic studies could allow better understanding of the role of genetics, genomics, epigenetics, and environment in the development of ASD. Post mortem brain tissue from affected individual could provide valuable materials for structural analysis as well as DNA and RNA expression studies. Coding and non-coding expression patterns and methylation signals may be useful for a better understanding of the role of genetics in ASD, particularly with a large number of patients sharing similar genetic backgrounds, patterns, and ethnicity. This improves the ability to identify large-effect pathogenic variants for facilitating genotype–phenotype correlations and allow comparisons. These analytical genetic studies are also useful for detecting the biological processes, molecular functions with gene-interactions, and pathways that are more autism-specific. In the original thesis plan, we proposed to order brain tissue from UK brain bank with different parts of the brains (frontal lobe and cerebellum) from normal individuals and ASD patients for comparing the expression of candidate genes. Unfortunately, only ten samples from five normal individuals and two samples from ASD patients (two brain areas in each person) were able to be acquired. This was considered insufficient for meaningful data analysis but a further study should be considered.

Improved classification of how variants like missense or nonsense mutations may confer different effects at the protein level will be useful. It is possible that particular variants which code for specific

amino acids may affect more important protein regions or domains depending on amino acid positions. For example, some amino acids may confer mild consequences while others playa more important role in protein function. The correlation between gene variants and protein function will require more studies.

Increasing awareness and knowledge about ASD and commonly related neurobehavioral conditions with the contribution of genetic differences will be especially useful for healthcare professionals who provide evaluation and treatment services for ASD. This will also be meaningful for individuals living with ASD, their families and possibly for the benefit of society overall. Early recognition, diagnosis, and treatment could help individuals with ASD achieve optimal long-term outcomes and improved quality of life. Understanding of genetic patterns which could affect treatment outcomes, for example medications or stem cell transplantation, will help to improve the overall health of patients. More effective treatment interventions need further investigation and attention to become a reality.

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APPENDIX 1: Supplementary table

Supplementary table 1: Clinical details of all 100 ASD patients

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD002/ M	2007	36	108	1-2	22/12	41	C-section (3.3kg)/Walking at 12 m/o
ASD003/ M	1999	30	209	3	21/12	50	Vaginal
ASD004/ M	2010	36	89	3	20/12	50	C-section (2.8kg)/IVF twins, sibling with ASD, walking at 14 m/o
ASD005/ M	2013	36	45	3	24/12	50	C-section (3.8kg), mother was 42 y/o when delivered/Walking at 24 m/o
ASD006/ F	2011	18	78	2-3	21/12	47	C-section (3.4kg)/Walking and babbling at 13 m/o, language and intellectual regression after 18 m/o
ASD008/ F	2013	30	53	2	17/12	37	Vaginal (3.5kg)/Walking at 16 m/o, constipation

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD009/ M	2010	30	88	3	23/12	51	C-section (3.5kg)/ID
ASD010/ M	2009	36	93	2	18/12	35	Vaginal (3.2kg)/Walking and babbling at 12 m/o, language regression after 2.5 y/o, constipation
ASD011/ M	2011	24	74	3	21/12	47	Vaginal (3.6kg)/Babbling at 7-8 m/o, walking at 10 m/o, language regression after 18 m/o
ASD012/ M	2003	18	161	2	20/12	43	C-section (3.2kg)/Walking at 12 m/o, speech delay
ASD013/ M	2012	24	60	3	21/12	51	C-section (3.2kg)/Babbling at 10 m/o, walking at 13.5 m/o, language regression after 14-15 m/o
ASD014/ M	2004	n/a	148	n/a	n/a	n/a	Vaginal (3.5kg)/
ASD015/ M	2012	24	60	3	22/12	53	Vaginal (1.7kg)/Preterm birth at 7th months of gestation, walking at age of 3, ID, DD

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD016/ M	2011	22	67	3	21/12	48	Preterm birth at 33 weeks of gestation (1.9kg), GI problems
ASD017/ F	2006	18	126	n/a	n/a	53	Vaginal (3.2kg)/Walking at 12 m/o
ASD018/ M	2003	18	160	3	21/12	49	Vaginal (3.8kg)
ASD019/ M	2012	24	49	2	20/12	37	C-section (3.6kg)/Walking at 12 m/o, GI problems
ASD020/ M	2013	18	38	3	21/12	46	Vaginal (3.7kg)/walking and babbling at 13 m/o
ASD021/ M	2011	24	67	3	22/12	50	C-section (3.1kg)/Walking at 22 m/o
ASD022/ M	2011	18	72	3	20/12	55	C-section (3.2kg)/Walking at 15 m/o, speech delay
ASD023/ M	2012	27	57	2-3	18/12	44	Vaginal (3.4kg) at 41 weeks of gestation/Walking at 18 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD024/ F	2011	21	75	3	20/12	55	Vaginal preterm birth at 37 weeks of gestation (2.2kg)/Walking at 14 m/o
ASD025/ F	2011	24	61	3	17/12	46	Vaginal (2.8kg)/Flat feet, motor delay, ID
ASD026/ M	2010	30	79	3	22/12	45	Vaginal (3.2kg)/Walking at 14 m/o
ASD027/ M	2009	22	92	3	22/12	47	C-section (2.4kg)/Monozygotic twins, the sibling with ASD, walking at 15 m/o and babbling at 16 m/o, language and social regression
ASD028/ M	2007	38	113	3	20/12	42	Vaginal (3.3kg), mother was 37 y/o/Language regression after 14 m/o
ASD029/ M	2012	48	57	3	23/12	49	C-section (3.2kg)/Walking at 13-14 m/o
ASD030/ F	2010	22	79	2	22/12	37	C-section (3.8kg)/Walking and babbling at 12 m/o; Language regression after 18 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD031/ M	2011	20	75	3	22/12	55	Vaginal (3.8kg)/Walking at 12 m/o
ASD032/ M	2010	14	85	2	17/10	42	Vaginal (3.8kg)/Walking at 13 m/o, EP, constipation
ASD033/ M	2010	24	76	2	23/13	45	C-section (3.5kg)/Babbling at 10 m/o, walking at 12 m/o, language regression after 12 m/o
ASD034/ M	2011	21	77	3	18/12	50	Vaginal (3.3kg)/Babbling at 9 m/o, walking at 14 m/o, language regression after 17 m/o
ASD035/ M	2009	36	99	3	20/12	45	Forceps delivery/Walking at 17 m/o, babbling at 24 m/o
ASD036/ F	2009	18	57	3	20/12	49	Vaginal (3.5kg)/DD
ASD037/ M	2012	24	52	3	52	51	Vaginal (3.2kg), preterm birth at 32 weeks of gestation/ Babbling at 10 m/o and walking at 16 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD038/ M	2010	24	48	n/a	22/12	48	C-section (2.6kg)/Missed rolling and clawing, walking at 2 y/o; CP, ID
ASD039/ M	2010	18	81	3	22/12	53	Vaginal (3.5kg)/Babbling at 12 m/o, walking at 15 m/o
ASD040/ M	2011	20	70	3	22/12	52	Vaginal (3.2kg)/ Walking and babbling at 12 m/o, language regression after 18 m/o
ASD041/ M	2003	36	169	3	21/12	49	C-section (3.6kg)/Walking at 17-18 m/o, speech delay
ASD042/ M	2011	36	71	3	22/12	48	Vaginal (3kg)/Walking at 18 m/o, speech delay
ASD043/ M	2003	36	166	2	22/12	44	Vaginal (3.3kg)/Walking at 15 m/o, sensation disorders
ASD044/ M	2012	24	60	3	20/12	53	C-section (3.2kg)/Missed crawling, walking at 21 m/o, language regression after 7-8 m/o, EP at 12 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD045/ M	2011	27	64	3	24/12	50	Vaginal (3.4kg)/Walking at 14 m/o, speech delay
ASD046/ M	2006	24	152	3	21/12	46	Vaginal (3.2kg)/Babbling at 18-20 m/o, walking at 3 y/o, language regression after 2 y/o, motor delay
ASD047/ M	2009	18	90	3	22/12	50	C-section (2.9kg)/Babbling at 7-8 m/o, walking at 28 m/o, ID, disrupted sleep
ASD048/ M	2007	18	119	2	20/12	40	Vaginal (3.7kg)/Walking at 13 m/o, sensation disorders
ASD049/ M	2007	24	115	3	21/12	46	C-section (3.7kg)/Walking at 12-13 m/o, language regression after 18 m/o
ASD050/ M	2011	36	66	3	22/12	52	C-section (3kg), mother was 40 y/o when delivered/Babbling at 11 m/o, walking at 17 m/o
ASD051/ F	2007	24	112	1-2	n/a	35	Vaginal (2.6kg)/Walking at 12 m/o, babbling at 24 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD052/ M	2014	18	30	2	20/12	n/a	Vaginal (3.7kg)/Walking at 13 m/o, talking at 30 m/o, DD
ASD053/ M	2010	30	86	3	21/12	46	Vaginal (3.5kg)/Walking and babbling at 11 m/o
ASD054/ M	2011	24	76	3	16/12	50	C-section (2.7kg), mother was infected with rubella at 4th months of gestation/Walking at 18 m/o, babbling at 36 m/o, sleep deprivation, ID
ASD056/ M	2007	36	119	2	17/12	40	Vaginal (3.4kg)/Walking at 17-18 m/o and babbling at 24 m/o, language regression after 24 m/o
ASD057/ M	2011	36	70	3	20/12	48	C-section (2.5kg)/Walking at12 m/o, speech delay
ASD058/ M	2012	24	59	3	20/12	50	Vaginal (4.2kg)

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD059/ M	2010	22	80	2-3	21/12	46	C-section (3kg)/Sensation disorders
ASD060/ M	2012	16	54	2	15/12	38	Vaginal (3.4kg)/Walking at 12 m/o, talking at 24 m/o
ASD061/ M	2013	18	49	3	21/12	45	C-section (3.4kg)/Walking at 14 m/o, babbling at 9 m/o, language regression after 12 m/o
ASD062/ M	2011	28	74	3	18/12	46	Vaginal (3.3kg)/2nd child, older brother presented speech delay, walking at 12 m/o, sleep disorder
ASD063/ M	2012	36	56	3	20/12	45	C-section (4.1kg)/Walking at 15 m/o
ASD064/ M	2007	24	117	2	15/12	40	C-section (3.4kg)/Walking at 14-15 m/o, speech delay
ASD065/ F	2010	83	83	2-3	22/12	43	C-section (3.35kg)/Older brother with CP, walking and babbling at 12 m/o, language

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
							regression after 12 m/o, EP at 8 m/o, sensation disorders, DD
ASD066/ M	2012	24	52	3	21/12	50	C-section (2.9kg), mother was 43 y/o when delivered/Walking at 16 m/o
ASD067/ M	2009	18	98	3	22/12	46	Vaginal (3.2kg)/Walking at 14 m/o, babbling at 20 m/o, sleep disorder
ASD068/ F	2013	18	44	3	20/12	54	Vaginal (4.2kg)/Walking at 12 m/o, EP
ASD069/ M	2011	24	73	3	22/12	49	Vaginal (3.1kg), mother was 39 y/o when delivered/Walking at 15 m/o, talking at 48 m/o
ASD070/ M	2013	18	42	3	20/12	49	Vaginal (2.9kg)/Walking and babbling at 12 m/o, language regression after 16 m/o
ASD071/ M	2012	36	59	3	20/12	53	C-section (3kg), mother was under depression treatment during pregnancy/Walking at 22

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
							m/o, babbling at 12 m/o, language regression after 18 m/o
ASD072/ M	2012	24	59	3	20/12	56	Vaginal (1.3kg), preterm birth at 7th month of gestation/Older brother with speech delay; Walking at 15 m/o, babbling at 18 m/o, asthma
ASD073/ F	2012	24	55	3	20/12	50	C-section (3.9kg)/Walking at 9 m/o
ASD074/ M	2009	21	95	2-3	21/12	39	Vaginal (3.4kg) at 37 weeks of gestation/ Visual defect, walking at 18 m/o, talking at 12 m/o, sleep deprivation
ASD075/ M	2012	24	59	3	19/12	51	C-section (3.1kg), birth asphyxia/Older brother with speech delay and ID
ASD076/ M	2011	24	70	3	20/12	54	C-section (3.7kg)/Walking at 14 m/o, babbling at 12 m/o, language regression

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD077/ M	2011	30	71	2-3	18/12	42	C-section at 41 weeks of gestation (3.6kg)/Walking at 13 m/o, babbling at 24 m/o
ASD078/ M	2014	18	36	3	16/12	45	Emergency C-section at 38 weeks of gestation (3.5kg) due to intrauterine growth restriction
ASD079/ M	2011	20	70	3	24/12	51	C-section (3.6kg)/ Walking at 12 m/o
ASD080/ F	2012	48	66	3	21/12	50	Vaginal/Walking at 2 y/o, disrupted sleep
ASD081/ M	2013	19	54	3	20/12	49	C-section at 37 weeks of gestation (3.7kg)/Walking at 13 m/o, talking at 13 m/o, language regression after 17-18 m/o
ASD082/ F	2012	24	62	3	n/a	45	C-section (2.9kg)/ Congenital foot deformity, walking at 19-20 m/o

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD083/ M	2011	26	50	2-3	20/12	46	C-section (3.2kg)/ Walking and babbling at 9 m/o
ASD084/ M	2013	24	53	3	22/12	46	Vaginal (3.3kg)/Walking at 13 m/o, babbling at 18 m/o, language delay after 18 m/o
ASD085/ M	2013	18	42	3	20/12	44	Vaginal at 35 weeks of gestation (2.4kg)/Walking at 12 m/o
ASD086 M	2012	17	57	2-3	21/12	45	C-section (4kg)/Walking at 12 m/o, speech delay
ASD087/ M	2010	36	88	3	23/12	44	Vaginal (3.3kg)
ASD088/ M	2014	39	41.5	2-3	20/12	39	C-section at 36 weeks of gestation (3.6kg)/Walking at 13 m/o, babbling at 9 m/o, often constipation
ASD089/ M	2012	36	62	3	20/12	54	Vaginal (3.3kg)/Walking at 24 m/o, ID, decrease perception of pain

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD090/ M	2010	24	80	3	18/12	49	Vaginal (3.5kg)/Walking at 10 m/o
ASD091/ F	2009	21	99	3	22/12	51	Vaginal preterm birth at 36 weeks of gestation (3.8kg), ID
ASD092/ M	2012	24	63	3	19/12	53	C-section (3.9kg)/ Walking at 14 m/o, babbling at 10 m/o, language regression
ASD093/ M	2008	22	109	3	19/12	45	C-section (3kg)/ Walking at 17 m/o, babbling at 18-19 m/o
ASD094/ M	2009	30	95	1-2	15/12	41	Vaginal, no concern at birth
ASD095/ M	2012	18	62	3	22/12	53	Vaginal (3kg)/Chronic constipation, malnutrition
ASD096/ M	2009	26	87	3	22/12	48	C-section (3.6kg)/Normal motor milestones (crawling, walking)
ASD097/ M	2004	24	154	3	22/12	50	Vaginal (3.2kg), mother was 35 y/o/Walking at 24 m/o, speech delay, sleep deprivation, ID

Proband / Gender	**DOB**	**Diagnosis**					**Clinical features**
		Age when diagnosed (month)		DSM-V level	ADOS score	CARS score	Delivery (weight at birth)/ Milestones & other presentations
		First	Enrollin				
ASD098/ F	2009	18	88	n/a	n/a	4 1	Vaginal (3.2kg), full term/ Rolling at 5 m/o, walking at 15 m/o, babbling at 14 m/o, language and social interaction regressed after 16 m/o
ASD099/ M	2008	24	66	3	21/12	4 8	Vaginal at 38 weeks of gestation (3.7kg)/Walking at 14 m/o, babbling at 12 m/o, sleep disorder, Chiari I, ID
ASD100/ F	2008	36	95	n/a	n/a	4 8	C-section (3.4kg), mother was 36 y/o/Walking at 12 m/o
ASD101/ M	2011	24	68	3	n/a	4 8	Vaginal, birth asphyxia/Walking at 30 m/o, ID
ASD102/ M	2014	19	35	2-3	22/12	4 4	C-section (2.6kg) at 37 weeks of gestation/Walking at 19 m/o, speech delay
ASD103/ M	2009	36	96	2-3	22/12	4 3	C-section (3.1kg)/Walking at 12 m/o, speech delay

Supplementary table 2: Brain imaging information in probands

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD002/M	n/a	NAD
ASD003/M	n/a	n/a
ASD004/M	Parietal lobe, central sulcus, hippocampus, anterior cingulate gyrus	n/a
ASD005/M	n/a	n/a
ASD006/F	Temporal lobe, hippocampus, right parietal lobe	n/a
ASD008/F	n/a	n/a
ASD009/M	n/a	n/a
ASD010/M	n/a	n/a
ASD011/M	Hippocampus, central sulcus, parietal lobe, anterior cingulate gyrus	n/a
ASD012/M	n/a	n/a
ASD013/M	Temporal lobe, hippocampus, cerebellum	n/a
ASD014/M	n/a	n/a
ASD015/M	n/a	ABN
ASD016/M	n/a	n/a
ASD017/F	n/a	ABN
ASD018/M	n/a	n/a
ASD019/M	n/a	n/a
ASD020/M	n/a	n/a

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD021/M	Anterior cingulate gyrus	n/a
ASD022/M	Temporal lobe, bilateral hippocampus, prefrontal cortex	NAD
ASD023/M	n/a	n/a
ASD024/F	Parietal lobe, central sulcus, temporal lobe, hippocampus, anterior cingulate gyrus	ABN
ASD025/F	n/a	ABN
ASD026/M	n/a	n/a
ASD027/M	n/a	n/a
ASD028/M	n/a	NAD
ASD029/M	n/a	ABN
ASD030/F	n/a	NAD
ASD031/M	Parietal lobe, central sulcus, temporal lobe, hippocampus, anterior cingulate gyrus	n/a
ASD032/M	n/a	n/a
ASD033/M	n/a	n/a
ASD034/M	Hippocampus, anterior cingulate gyrus	NAD
ASD035/M	n/a	ABN
ASD036/F	n/a	NAD
ASD037/M	n/a	n/a
ASD038/M	n/a	NAD

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD039/M	n/a	NAD
ASD040/M	Parietal lobe, anterior cingulate gyrus, hippocampus	n/a
ASD041/M	n/a	n/a
ASD042/M	n/a	n/a
ASD043/M	n/a	n/a
ASD044/M	Parietal lobe, central sulcus, temporal lobe, hippocampus, anterior cingulate gyrus	NAD
ASD045/M	Temporal lobe, hippocampus, anterior cingulate gyrus	ABN
ASD046/M	n/a	NAD
ASD047/M	n/a	NAD
ASD048/M	n/a	n/a
ASD049/M	n/a	n/a
ASD050/M	n/a	n/a
ASD051/F	n/a	ABN
ASD052/M	n/a	n/a
ASD053/M	n/a	n/a
ASD054/M	n/a	ABN
ASD056/M	n/a	NAD
ASD057/M	n/a	n/a
ASD058/M	n/a	n/a

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD059/M	n/a	NAD
ASD060/M	n/a	n/a
ASD061/M	n/a	n/a
ASD062/M	Parietal lobe, central sulcus, hippocampus, anterior cingulate gyrus	n/a
ASD063/M	Hippocampus, frontal lobe	NAD
ASD064/M	n/a	n/a
ASD065/F	n/a	n/a
ASD066/M	n/a	n/a
ASD067/M	n/a	n/a
ASD068/F	n/a	NAD
ASD069/M	Hippocampus, anterior cingulate gyrus	NAD
ASD070/M	Central sulcus, hippocampus	n/a
ASD071/M	Central sulcus, hippocampus, anterior cingulate gyrus	NAD
ASD072/M	Parietal lobe, hippocampus, anterior cingulate gyrus	NAD
ASD073/F	Temporal lobe, hippocampus, anterior cingulate gyrus	n/a
ASD074/M	n/a	ABN
ASD075/M	Hippocampus, parietal lobe, anterior cingulate gyrus	n/a
ASD076/M	Hippocampus, frontal lobe	n/a
ASD077/M	Hippocampus, anterior cingulate gyrus	n/a

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD078/M	n/a	n/a
ASD079/M	Parietal lobe, hippocampus, anterior cingulate gyrus	n/a
ASD080/F	Frontal lobe, anterior cingulate gyrus, hippocampus	n/a
ASD081/M	Hippocampus, anterior cingulate gyrus	n/a
ASD082/F	n/a	n/a
ASD083/M	n/a	n/a
ASD084/M	n/a	n/a
ASD085/M	n/a	NAD
ASD086M	n/a	n/a
ASD087/M	n/a	ABN
ASD088/M	n/a	n/a
ASD089/M	n/a	ABN
ASD090/M	Anterior cingulate gyrus, hippocampus	n/a
ASD091/F	n/a	NAD
ASD092/M	n/a	ABN
ASD093/M	n/a	NAD
ASD094/M	n/a	n/a
ASD095/M	Central sulcus, hippocampus, anterior cingulate gyrus	n/a
ASD096/M	n/a	NAD
ASD097/M	n/a	NAD

Proband/ Gender	**Brain imaging**	
	FDG-PET/CT (FDG hypometabolism)	MRI
ASD098/F	n/a	ABN
ASD099/M	n/a	ABN
ASD100/F	n/a	NAD
ASD101/M	Hippocampus, anterior cingulate gyrus	n/a
ASD102/M	Hippocampus, anterior cingulate gyrus	n/a
ASD103/M	n/a	NAD

Supplementary Table 3: Details of clinical features

Social interaction, language, repetitive and restricted activities and interests are clinical criteria listed in 100 patients divided in male and female

Domain	**Male** **N**	**Female** **N**
Social interaction		
No social interaction	24	3
Have social interaction	59	14
Eye contact		
No eye contact	16	2
Little and normal eye contact	67	15
Expression of feeling to parent		
Showing affection	66	14
No showing affection	17	3
Expressive language		
No language	35	6
Single word	36	6
Sentence	12	5
*Stereotypic/**Repetitive Behaviors***		
Much repetitive behavior	54	5
Less repetitive behavior	29	12
No repetitive behavior	0	0
Restricted interests		
Much restricted interest	59	5

Less restricted interest	33	12
No restricted interest	1	0
Hyperactivity		
Have hyperactivity	53	12
No hyperactivity	20	5
Self-injurious behavior		
Have self-injurious behavior	12	3
No self-injurious behavior	71	14
Sensory Impairments		
Have sensory abnormalities	69	9
No sensory abnormalities	14	8
Picky eating behaviours		
Have picky eating behavior	38	5
No picky eating behavior	45	12
Sleep problems		
Have sleep problems	16	2
No sleep problems	67	15
Self-feeding		
Unable to self-feed	11	3
Need support to self-feed	35	3
Able to self-feed	37	11
Toileting skills		
Unable to go to toilet	23	4

Need support to go to toilet	27	2
Able to go to toilet	33	11
Learning ability		
Unable to integrate into school	68	11
Need support to integrate into school (with help from teachers)	14	5
Go to school normally	1	1
Gut issue		
Have gut issue	4	0
Normal	79	17

Supplementary Table 4: Details of 118 unique CNVs

No	Chr	Position	Start	End	Probes	DLRS	P value	Genes
1	chr1	p22.3	87099791	87105798	3	-1.01571	9.49E-12	*LOC105378828, CLCA3P*
2	chr1	p36.13	16927124	17231817	12	0.492793	6.06E-16	*NBPF1, CROCCP2, MST1P2, FAM231AP, FAM231A, FAM231C, MIR3675, ESPNP, MST1L, LOC102724562, LOC440570, LOC105376805*
3	chr1	p32.1	59842022	59878289	8	-0.52676	9.62E-10	*FGGY*
4	chr1	p22.1	93002721	93031671	6	-1.07901	1.16E-24	*EVI5*
5	chr1	p36.11	25675212	25688208	4	0.98616	1.02E-20	*TMEM50A*
6	chr1	p36.22	10505050	10516509	4	0.680744	1.63E-10	*CENPS-CORT, CORT*
7	chr1	q21.3	152766564	152773898	3	-0.88721	7.34E-12	*LCE1D*
8	chr1	q31.3	196705001	196781644	6	-0.60548	2.27E-10	*CFH, CFHR3*
9	chr1	q44	248601802	248646776	7	0.520891	1.88E-10	*OR2T7, OR2T2, OR2T3*
10	chr1	p36.33	1585691	1613809	9	0.483988	3.05E-12	*CDK11B, SLC35E2B*
11	chr1	p36.13	17207777	17276064	9	-0.71563	1.45E-20	*LOC105376805, CROCC*
12	chr1	q42.13	228783832	228788356	3	-0.96754	3.39E-14	*RHOU, DUSP5P1*
13	chr1	q24.2	167638467	167646427	3	-0.76556	6.47E-10	*RCSD1*
14	chr1	p36.23	8672082	8688845	3	-1.02107	7.65E-15	*RERE*
15	chr1	p13.2	111832138	111835175	3	-0.95271	8.55E-14	*CHIA*
16	chr1	q21.1	144986366	145076206	21	-0.3907	4.07E-18	*NBPF20, NBPF19, NBPF9, LOC100996724, PDE4DIP*
17	chr1	q42.2	231535636	231542955	3	-0.77262	4.08E-10	*EGLN1*
18	chr2	q11.2	100,758,125	100,765,082	3	-0.80014	1.36E-10	*AFF3*
19	chr2	q37.3	242,750,401	242,758,759	3	0.845233	1.10E-11	*NEU4*
20	chr2	p13.3	71,337,169	71,354,407	4	-0.66806	5.02E-10	*MCEE*
21	chr3	p21.31	48,580,689	48,680,593	20	0.25401	1.62E-10	*PFKFB4, MIR6823, UCN2, COL7A1, MIR711, UQCRC1, SNORA94, TMEM89, SLC26A6, MIR6824, CELSR3*
22	chr3	q25.32	157,850,581	157,870,391	5	-0.61068	9.18E-10	*RSRC1*
23	chr4	q31.21	144,805,113	145,057,804	18	0.439419	1.12E-15	*GYPE, LOC101927636, GYPB, GYPA*
24	chr4	q32.3	166,334,022	166,351,395	5	-0.62225	2.85E-11	*CPE*
25	chr4	q32.2	162,474,015	162,488,007	4	0.689832	7.47E-10	*FSTL5*
26	chr4	p16.1	6,401,138	6,413,973	4	-0.5961	2.48E-10	*PPP2R2C*
27	chr4	q31.21	144,986,413	145,032,578	6	0.565563	2.85E-10	*GYPA*
28	chr4	q22.2	93,919,081	93,969,117	10	-0.70495	6.01E-24	*GRID2*
29	chr4	q34.1-q35.2	175,897,368	190,896,674	1847	-0.78104	4.900e-324	*ADAM29, GPM6A, LOC101928590, WDR17, SPATA4, ASB5, SPCS3, VEGFC,*

								LINC02509, NEIL3, AGA, LINC01098, LINC01099, LINC00290, LINC02500, TEMN3-AS1, LOC90768, MIR1305, TENM3, DCTD, FAM92A1P2, WWC2-AS2, WWC2, WWC2-AS1, CLDN22, CLDN24, CDKN2AIP, LOC389247, ING2, RWDD4, TRAPPC11, STOX2, ENPP6, LINC02363, LINC02362, IRF2, LINC02427, LINC02365, CASP3, PRIMPOL, CENPU, ACSL1, SLED1, MIR3945HG, MIR3945, LINC01093, MIR4455, HELT, LINC02436, SLC25A4, CFAP97, SNX25, LRP2BP, ANKRD37, UFSP2, C4orf47, CCDC110, LOC105377590, PDLIM3, SORBS2, TLR3, FAM149A, FLJ38576, CYP4V2, KLKB1, F11, F11-AS1, MTNR1A, FAT1, LINC02374, LOC339975, LINC02514, LINC02492, ZFP42, TRIML2, TRIML1, LINC01060, LINC02508, LINC01262, LOC105379514, FRG1-DT, LINC01596, FRG1
30	chr5	p15.33	685,256	777,000	5	0.665994	8.79E-10	*TPPP, ZDHHC11B*
31	chr5	q11.2	51,335,134	51,426,885	5	0.827911	1.73E-10	*LINC02118*
32	chr5	p15.33 - p15.32	1,910,175	5,391,389	451	0.305105	5.55E-28	*LOC100506858, LSINCT5, IRX2, C5orf38, LOC105374620, LINC01377, LINC01019, LINC02162, LINC01017, IRX1, LINC02114, LINC01020, LINC02121, CTD-2297D10.2, ADAMTS16*
33	chr5	q31.3	140,210,459	140,216,357	3	-0.98042	1.76E-11	*PCDHA1, PCDHA2, PCDHA3, PCDHA4, PCDHA5, PCDHA6, PCDHA7*
34	chr5	q13.2	68,818,173	70,657,747	8	-0.67097	6.85E-15	*OCLN, SNORD13B-2, SNORD13B-1, GTF2H2C_2, GTF2H2C, GUSBP3, LOC653080, SERF1A, SERF1B, SMN2, SMN1, SMA4, GTF2H2B, SMA5, LOC441081, GUSBP9, NAIP, GTF2H2, LOC647859, LINC02197*
35	chr5	q14.3	90,077,035	90,083,071	3	0.836005	5.95E-10	*ADGRV1*
36	chr5	q13.2	68,815,067	68,824,041	3	-0.78937	5.17E-10	*OCLN*
37	chr6	p21.31	35,745,231	35,765,416	4	0.700719	5.04E-10	*CLPSL2, CLPSL1, CLPS*
38	chr6	p25.2	3,719,574	3,727,801	3	-0.86386	8.63E-12	*PXDC1*
39	chr6	q14.3	86,373,607	86,395,961	5	0.69426	1.90E-13	*SNHG5, SNORD50A, SNORD50B*
40	chr6	p22.1	29,913,929	29,945,434	6	-0.55782	5.98E-11	*HCG9*
41	chr7	q35	143,425,418	143,466,132	5	0.643663	1.00E-10	*TCAF2, CTAGE6*
42	chr7	q34	142,340,360	142,497,079	26	-0.44212	2.92E-25	*MTRNR2L6, PRSS1, PRSS3P2*
43	chr7	q35	147,531,869	147,551,624	5	-0.51394	1.63E-10	*CNTNAP2*

44	chr7	q35	143,951,931	144,074,551	4	-0.6545	2.44E-10	*LOC101928605, OR2A1-AS1, OR2A7, ARHGEF34P, CTAGE4, CTAGE8, OR2A20P, OR2A9P, OR2A1, OR2A42, ARHGEF5*
45	chr7	q22.1	100,333,268	100,341,714	3	-0.80157	2.70E-10	*ZAN*
46	chr7	q35	143,466,073	143,547,906	3	-0.87333	3.91E-10	*TCAF2P1, LOC154761*
47	chr7	q35	143,257,517	143,434,537	5	-0.93277	2.14E-20	*CTAGE15, TCAF2, TCAF2P1*
48	chr8	p22	16,954,134	16,984,198	7	0.714118	3.56E-11	*MICU3*
49	chr8	p23.2	3,545,824	3,583,362	9	0.689289	3.49E-10	*CSMD1*
50	chr8	p23.1	10,403,206	10,416,312	3	-0.8661	3.34E-11	*PRSS55*
51	chr8	p12	32,421,470	32,438,790	5	-0.58294	1.45E-10	*NRG1*
52	chr9	p23	10,550,843	10,558,986	3	-0.78138	4.83E-11	*PTPRD*
53	chr9	q22.31	96,166,255	96,227,148	10	-0.55163	2.94E-10	*FAM120AOS, FAM120A*
54	chr9	q31.1	108,164,850	108,181,793	4	0.698271	1.46E-10	*SLC44A1*
55	chr9	q21.33	88,823,348	88,840,945	4	-0.89031	8.98E-18	*C9orf153*
56	chr9	q34.3	139,326,377	139,516,668	38	0.395903	1.25E-17	*INPP5E, SEC16A, C9orf163, NOTCH1, MIR4673, MIR4674, NALT1, LINC01451*
57	chr10	q25.1	108,453,644	108,473,165	4	0.768123	1.88E-10	*SORCS1*
58	chr10	p11.21	37,450,623	37,468,455	3	0.978304	3.56E-11	*ANKRD30A*
59	chr10	p15.3	1,518,099	1,525,945	3	-0.66806	3.93E-10	*ADARB2*
60	chr11	q13.2-q13.4	67,856,136	73,062,386	810	-0.86419	4.900e-324	*CHKA, KMT5B, C11orf24, LRP5, PPP6R3, GAL, TESMIN, CPT1A, MRPL21, IGHMBP2, MRGPRD, MRGPRF, MRGPRF-AS1, TPCN2, MIR3164, LOC338694, MYEOV, LOC102724265, LINC01488, CCND1, ORAOV1, FGF19, FGF4, FGF3, LOC101928443, ANO1-AS2, ANO1, FADD, PPFIA1, MIR548K, CTTN, SHANK2, SHANK2-AS1, SHANK2-AS3, MIR3664, FLJ42102, DHCR7, NADSYN1, MIR6754, KRTAP5-7, KRTAP5-8, KRTAP5-9, KRTAP5-10, KRTAP5-11, FAM86C1, ALG1L9P, ZNF705E, DEFB108B, LOC100133315, DEFB131B, RNF121, IL18BP, NUMA1, LOC100128494, MIR3165, LRTOMT, LAMTOR1, ANAPC15, FOLR3, FOLR1, FOLR2, INPPL1, PHOX2A, CLPB, LINC01537, PDE2A, MIR139, ARAP1, ARAP1-AS2, STARD10, MIR4692, ATG16L2, FCHSD2, MIR4459, P2RY2, P2RY6, LOC100287837, ARHGEF17*
61	chr11	p15.4	4,962,435	4,987,947	6	0.579683	1.64E-11	*OR51A4, OR51A2*
62	chr11	q14.3	89,856,654	89,885,908	6	0.68939	8.65E-11	*NAALAD2*

63	chr11	q22.3	107,655,060	107,673,765	5	-0.59082	4.68E-10	*SLC35F2*
64	chr11	q23.3	117,051,017	117,070,410	5	0.489845	8.70E-10	*SIDT2, LOC100652768, TAGLN*
65	chr11	q25	132,574,785	132,586,756	4	0.840603	5.45E-10	*OPCML*
66	chr11	p11.12	50,378,743	51,511,461	11	0.507499	1.95E-15	*LOC646813, OR4A5*
67	chr11	p14.3	24,924,128	24,971,889	11	0.645227	1.05E-11	*LUZP2*
68	chr11	p15.4	4,962,435	4,971,528	3	0.686155	1.01E-09	*OR51A4*
69	chr11	p15.1	16,917,227	16,925,760	3	-1.43093	1.26E-21	*PLEKHA7*
70	chr11	q14.1	78,368,953	78,399,909	7	-0.55249	3.23E-11	*TENM4*
71	chr12	p13.2	10,492,679	10,521,371	6	-0.68832	2.17E-13	*LOC101928100*
72	chr13	q12.3	29,749,504	29,759,979	3	0.838787	1.23E-10	*MTUS2*
73	chr14	q32.33	104,546,943	104,623,679	25	0.294784	1.19E-10	*ASPG, MIR203A, MIR203B, KIF26A*
74	chr14	q12	31,579,681	31,589,026	3	-0.82288	9.63E-10	*HECTD1*
75	chr14	q11.2	22,277,272	23,266,159	197	-0.29822	1.64E-87	*LOC105370401, LINC02332, DAD1, ABHD4, OR6J1, OXA1L, SLC7A7*
76	chr15	q11.2	25,430,535	25,499,179	14	0.505078	1.15E-10	*SNHG14, SNORD115-9, SNORD115-10, SNORD115-12, SNORD115-5, SNORD115-11, SNORD115-29, SNORD115-36, SNORD115-43, SNORD115-13, SNORD115-14, SNORD115-16, SNORD115-17, SNORD115-18, SNORD115-19, SNORD115-20, SNORD115-15, SNORD115-21, SNORD115-22, PWAR4, SNORD115-23, SNORD115-24, SNORD115-25, SNORD115-26, SNORD115-27, SNORD115-28, SNORD115-30, SNORD115-31, SNORD115-32, SNORD115-33, SNORD115-34, SNORD115-35, SNORD115-37, SNORD115-38, SNORD115-39, SNORD115-40, SNORD115-41, SNORD115-42, SNORD115-44*
77	chr15	q11.1-q11.2	20,432,851	22,835,945	71	-0.33186	3.18E-39	*CHEK2P2, HERC2P3, GOLGA6L6, GOLGA8CP, NBEAP1, MIR3118-2, MIR3118-3, MIR3118-4, POTEB, POTEB2, POTEB3, NF1P2, MIR5701-1, MIR5701-2, MIR5701-3, LINC01193, LINC02203, FAM30C, LOC646214, CXADRP2, LOC101927079, OR4M2, OR4N4, OR4N3P, IGHV1OR15-1,*

								LOC102724760, IGHV1OR15-3, LOC642131, MIR1268A, REREP3, MIR4509-1, MIR4509-2, MIR4509-3, GOLGA8DP, GOLGA6L1, GOLGA6L22, TUBGCP5
78	chr15	q24.3	76,883,703	76,893,443	3	0.810683	2.22E-10	*SCAPER*
79	chr15	q11.1-q13.3	20,102,541	32,445,252	1318	0.92734	4.900e-324	*CHEK2P2, HERC2P3, GOLGA6L6, GOLGA8CP, NBEAP1, MIR3118-2, MIR3118-3, MIR3118-4, POTEB, POTEB2, POTEB3, NF1P2, MIR5701-1, MIR5701-2, MIR5701-3, LINC01193, LINC02203, FAM30C, LOC646214, CXADRP2, LOC101927079, OR4M2, OR4N4, OR4N3P, IGHV1OR15-1, LOC102724760, IGHV1OR15-3, LOC642131, MIR1268A, REREP3, MIR4509-1, MIR4509-2, MIR4509-3, GOLGA8DP, GOLGA6L1, GOLGA6L22, TUBGCP5, CYFIP1, NIPA2, NIPA1, LOC283683, WHAMMP3, GOLGA8IP, HERC2P2, HERC2P7, GOLGA8EP, GOLGA8S, GOLGA6L2, MIR4508, MKRN3, MAGEL2, NDN, PWRN4, PWRN2, PWRN3, PWRN1, NPAP1, SNRPN, SNHG14, SNURF, SNORD107, PWARSN, PWAR5, SNORD64, SNORD108, PWAR6, SNORD109B, SNORD109A, SNORD116-1, SNORD116-2, SNORD116-3, SNORD116-9, SNORD116-4, SNORD116-5, SNORD116-7, SNORD116-6, SNORD116-8, SNORD116-10, SNORD116-11, SNORD116-12, SNORD116-13, SNORD116-14, SNORD116-15, SNORD116-16, SNORD116-19, SNORD116-17, SNORD116-18, SNORD116-20, SNORD116-21, SNORD116-22, SNORD116-23, SNORD116-24, SNORD116-25, SNORD116-26, SNORD116-27, SNORD116-28, SNORD116-29, SNORD116-30, IPW, PWAR1, SNORD115-1, SNORD115-2, SNORD115-3, SNORD115-4, SNORD115-5, SNORD115-9, SNORD115-10, SNORD115-12, SNORD115-6, SNORD115-7, SNORD115-8, SNORD115-11, SNORD115-29, SNORD115-36, SNORD115-43, SNORD115-13, SNORD115-14, SNORD115-16, SNORD115-17, SNORD115-18,*

								SNORD115-19, SNORD115-20, SNORD115-15, SNORD115-21, SNORD115-22, PWAR4, SNORD115-23, SNORD115-24, SNORD115-25, SNORD115-26, SNORD115-27, SNORD115-28, SNORD115-30, SNORD115-31, SNORD115-32, SNORD115-33, SNORD115-34, SNORD115-35, SNORD115-37, SNORD115-38, SNORD115-39, SNORD115-40, SNORD115-41, SNORD115-42, SNORD115-44, SNORD115-45, SNORD115-46, SNORD115-47, SNORD115-48, UBE3A, ATP10A, MIR4715, LINC02346, LINC00929, GABRB3, GABRA5, GABRG3, GABRG3-AS1, OCA2, HERC2, GOLGA8F, GOLGA8G, HERC2P11, HERC2P9, GOLGA8M, WHAMMP2, LOC100289656, PDCD6IPP2, APBA2, FAM189A1, NSMCE3, LOC100130111, TJP1, GOLGA8J, ULK4P3, GOLGA8T, LINC02249, CHRFAM7A, DNM1P50, GOLGA8R, LOC100288203, GOLGA8Q, ULK4P1, ULK4P2, GOLGA8H, ARHGAP11B, LOC100288637, HERC2P10, FAN1, MTMR10, TRPM1, MIR211, LINC02352, LOC283710, KLF13, OTUD7A, CHRNA7
80	chr16	p13.13	10,787,986	10,825,301	7	-0.59091	5.29E-10	*TEKT5*
81	chr17	q25.3	79,686,397	79,945,570	16	0.425878	1.22E-13	*SLC25A10, GCGR, MCRIP1, PPP1R27, P4HB, ARHGDIA, ALYREF, ANAPC11, PCYT2, NPB, SIRT7, MAFG, MAFG-AS1, PYCR1, MYADML2, NOTUM, ASPSCR1*
82	chr17	q21.31	44,144,904	44,796,816	35	-0.3532	5.69E-13	*KANSL1, KANSL1-AS1, ARL17B, LRRC37A, ARL17A, NSFP1, LRRC37A2, NSF*
83	chr17	p13.3	832,192	840,044	3	-0.76757	9.16E-10	*NXN*
84	chr18	q23	74,221,768	74,289,678	13	-0.59265	6.65E-19	*LINC00908*
85	chr18	q21.1	46,061,372	46,079,976	5	-0.64149	9.47E-11	*CTIF*
86	chr19	q13.41	53,318,222	53,365,432	9	0.471631	6.01E-10	*ZNF28, ZNF468, ZNF320*
87	chr19	p13.3	5,832,884	5,840,036	3	-0.8454	2.77E-10	*FUT6*
88	chr19	p13.2	12,538,730	12,545,513	3	0.790988	4.84E-10	*ZNF443*

89	chr19	p13.2	7,957,531	7,977,072	5	-0.64286	3.71E-11	*LRRC8E, MAP2K7*
90	chr19	q13.42	54,188,930	54,193,007	3	-0.70051	3.63E-10	*MIR519C, MIR1283-1*
91	chr19	q13.42	54,625,063	54,650,201	6	0.706203	8.71E-11	*PRPF31, CNOT3*
92	chr19	q13.41	52,127,719	52,148,589	4	-0.59823	6.31E-10	*SIGLEC5, SIGLEC14*
93	chr20	q13.12	44,307,046	44,315,147	3	-1.05459	6.37E-16	*WFDC10B*
94	chr20	p12.1	13,060,017	13,069,862	3	-0.78223	2.87E-10	*SPTLC3*
95	chr20	p12.1	14,774,913	15,190,652	80	-0.78202	8.94E-202	*MACROD2, MACROD2-AS1*
96	chr20	q13.12	45,764,881	45,809,227	10	-0.57259	2.11E-14	*EYA2, MIR3616*
97	chr21	q22.13	38,297,322	38,308,143	3	-0.81492	8.93E-10	*HLCS*
98	chr21	q22.3	44,949,257	45,071,430	25	-0.31169	3.03E-13	*HSF2BP, H2BFS, MIR6070*
99	chr21	q22.3	44,953,212	45,085,165	27	-0.25406	2.71E-10	*HSF2BP, H2BFS, MIR6070, RRP1B*
100	chr22	q11.21	18,889,039	18,984,519	14	0.672096	3.44E-32	*DGCR6, PRODH, DGCR5*
101	chr22	q13.31	45,579,409	45,583,183	3	-0.78205	3.00E-10	*NUP50, LOC105373064*
102	chr22	q13.33	50,346,151	50,361,832	4	0.767625	7.90E-13	*PIM3, MIR6821*
103	chr22	q12.2	30,138,103	30,151,739	4	0.655037	1.48E-10	*ZMAT5*
104	chr22	q13.32 - q13.33	48,746,241	51,178,264	488	-0.85405	4.900e-324	*FAM19A5, LOC284933, MIR4535, LINC01310, C22orf34, MIR3667, BRD1, ZBED4, ALG12, CRELD2, PIM3, MIR6821, IL17REL, TTLL8, MLC1, MOV10L1, PANX2, TRABD, SELENOO, TUBGCP6, HDAC10, MAPK12, MAPK11, PLXNB2, DENND6B, PPP6R2, SBF1, ADM2, MIOX, LMF2, NCAPH2, SCO2, TYMP, ODF3B, KLHDC7B, SYCE3, CPT1B, CHKB-CPT1B, CHKB, CHKB-AS1, MAPK8IP2, ARSA, SHANK3, LOC105373100, ACR*
105	chr22	q13.2	42,945,014	42,961,804	4	-0.84768	7.45E-13	*SERHL2*
106	chr22	q13.33	51,137,326	51,170,223	10	-1.04534	7.35E-47	*SHANK3*
107	chr22	q12.1	28,593,914	28,604,587	4	0.63623	1.00E-09	*TTC28*
108	chr22	q13.33	49,564,639	51,178,264	329	-0.90018	4.900e-324	*C22orf34, MIR3667, BRD1, ZBED4, ALG12, CRELD2, PIM3, MIR6821, IL17REL, TTLL8, MLC1, MOV10L1, PANX2, TRABD, SELENOO, TUBGCP6, HDAC10, MAPK12, MAPK11, PLXNB2, DENND6B, PPP6R2, SBF1,*

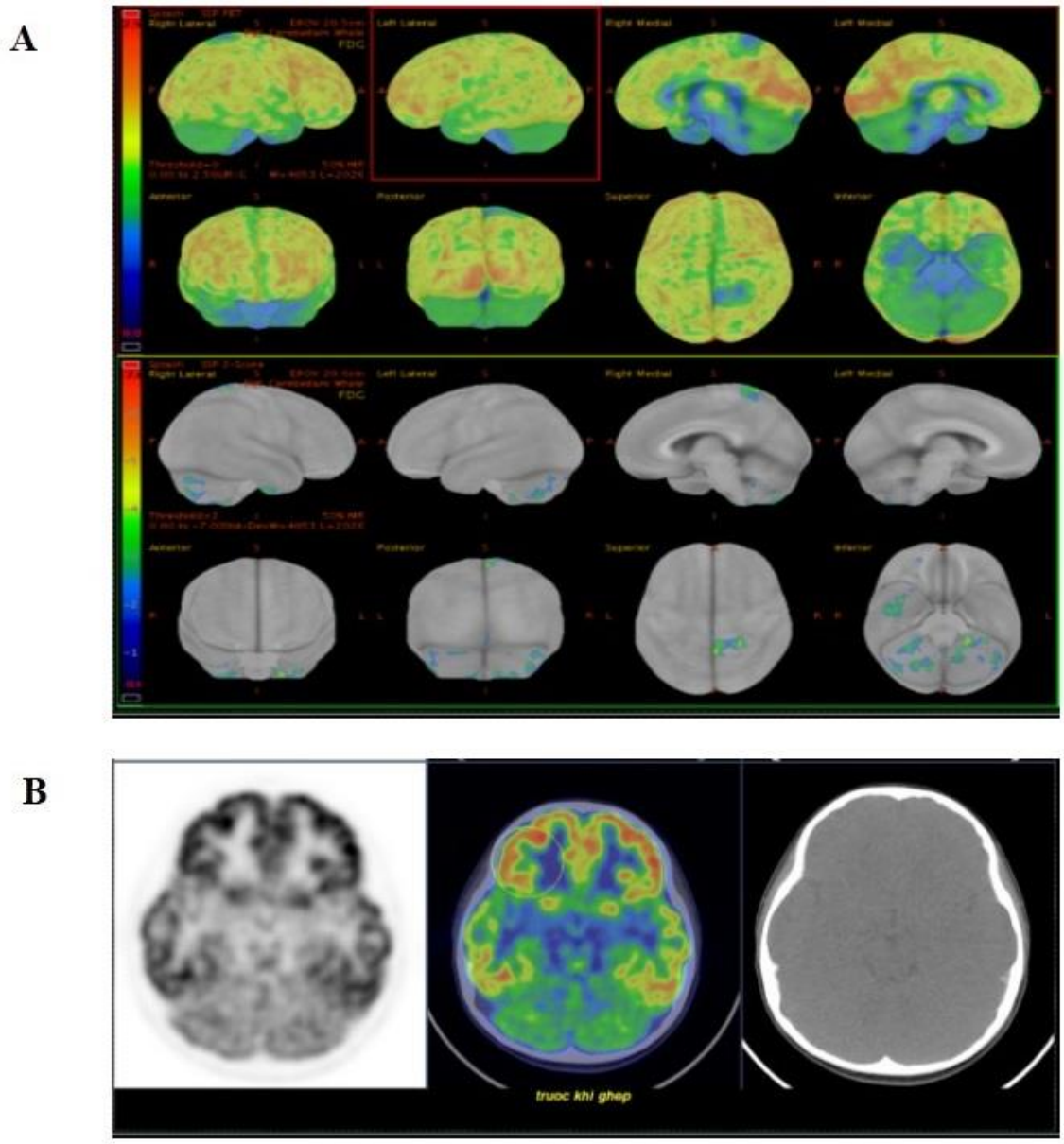
								ADM2, MIOX, LMF2, NCAPH2, SCO2, TYMP, ODF3B, KLHDC7B, SYCE3, CPT1B, CHKB-CPT1B, CHKB, CHKB-AS1, MAPK8IP2, ARSA, SHANK3, LOC105373100, ACR
109	chr22	q11.21	18,991,215	19,007,211	4	-0.79023	3.47E-12	*DGCR5, DGCR9*
110	chrX	p22.33	298,354	339,035	20	0.510428	3.99E-17	*PPP2R3B*
111	chrX	p22.33	70,397	505,732	113	0.312544	1.14E-15	*PLCXD1, GTPBP6, LINC00685, PPP2R3B*
112	chrX	p22.33	1,446,802	1,559,802	39	0.386651	1.52E-11	*IL3RA, SLC25A6, LINC00106, ASMTL-AS1, ASMTL*
113	chrX	p22.33	2,203,782	2,209,320	3	-0.68061	6.46E-10	*DHRSX*
114	chrX	p11.23	48,680,785	48,692,742	3	-1.04028	1.40E-10	*HDAC6, ERAS, PCSK1N*
115	chrX	p22.33	230,802	628,492	122	0.884013	4.900e-324	*GTPBP6, LINC00685, PPP2R3B, SHOX*
116	chrX	q28	153,387,892	153,505,604	7	-0.50813	4.64E-10	*OPN1LW, OPN1MW2, OPN1MW, OPN1MW3, TEX28*
117	chrY	p11.32	248,354	289,035	20	0.510428	2.40E-16	*PPP2R3B*
118	chrY	p11.2	9,641,061	9,650,194	3	1.282644	1.43E-15	*TTTY22*

Supplementary table 5: Primers sequence

Designing primers on one significant gene for confirmation of each candidate CNV. The internal control gene is VPS29.

Cytoband	Gene	Forward Primer	Reverse Primer
3p26.3	*CNTN6*	GTGGGTGTGTATAATAATGAAGG	CAAGGGAACACAAACTATGC
4q34.1-q35.2	*LRP2BP*	TGGTTGATGGAATTCCTTACCTT	AGCTGCAATGCCTTATCCAC
11q13.2-q13.4	*SHANK2*	GGGCTCAGACATGAACG	CTGGACAGCACGAACC
15q11.1-q13.3	*GABRB3*	CCTGAAGGGACTATAAGTGG	GTTACCGGAGTGGAAAGG
22q13.32-q13.33	*SHANK3*	AACTTCCATGACCCTGAC	TGGGTTCACAGACAGATAAG
12q24.11	*VPS29 (Reference gene)*	GTCCCGAGATAAGAATGTCC	CAGAAAGTTGTGACTGTTGG

APPENDIX 2: SUPPLEMENTARY FIGURES

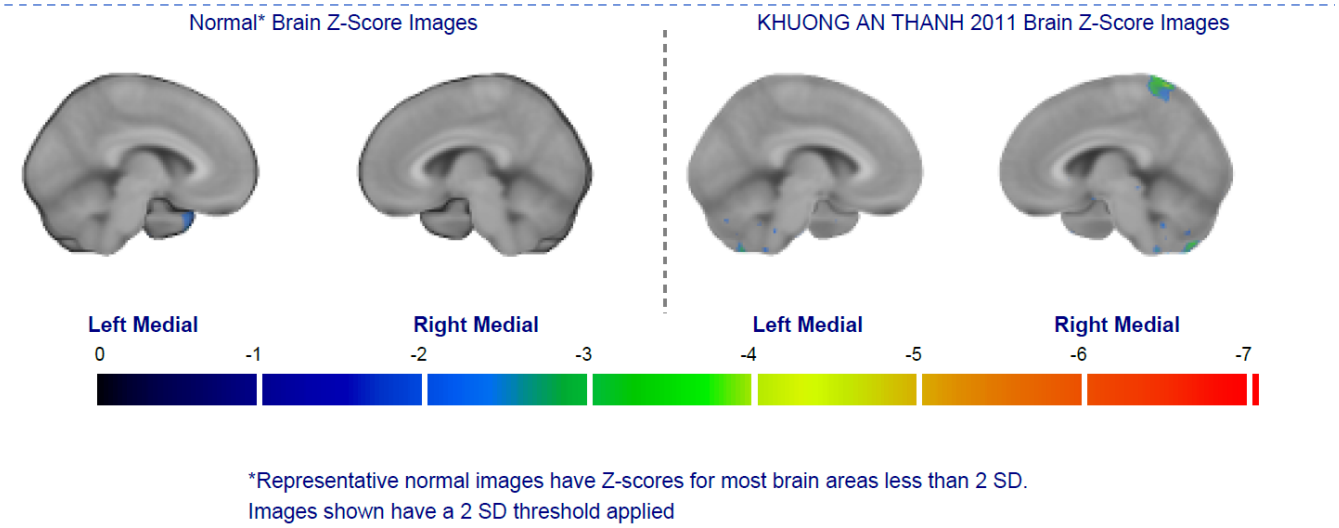


Supplementary figure 1: An example of FDG brain PET-CT images

The Positron emission tomography-computed tomography (PET/CT) images show hypometabolism in multiple areas of the brain of the ASD patient. Dark blue brain areas were defined as severe reductions in FDG metabolic rate and light blue brain areas were assessed as moderate metabolic rates. Green was considered to have mild metabolic reduction and the yellow-orange brain region was assessed as increased FDG metabolism.

Panel A. Hypometabolism in many areas of the patient's brain

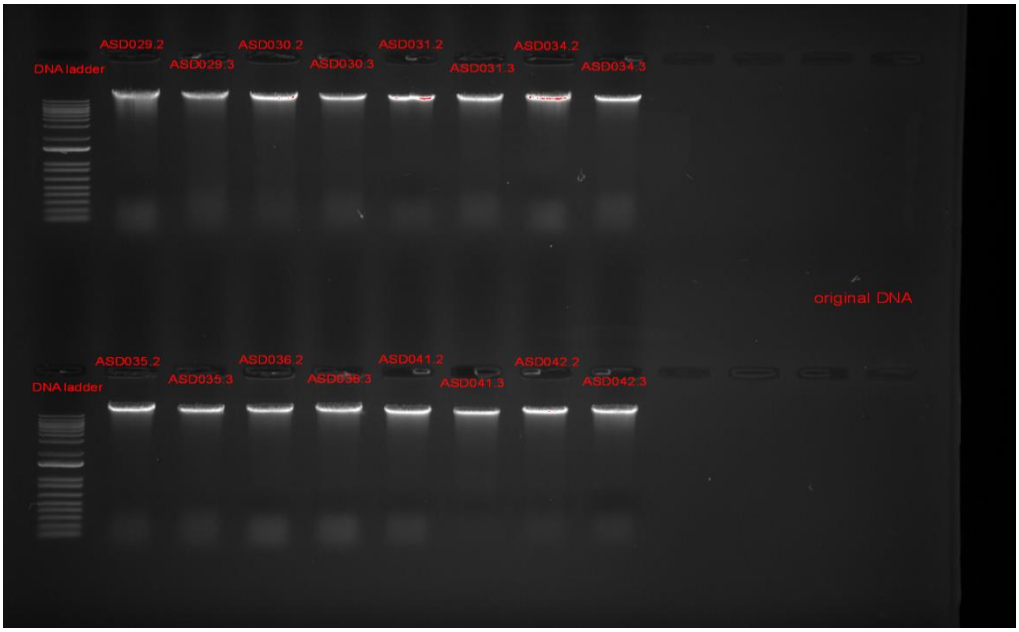
Panel B. Hypometabolism in the frontal lobe

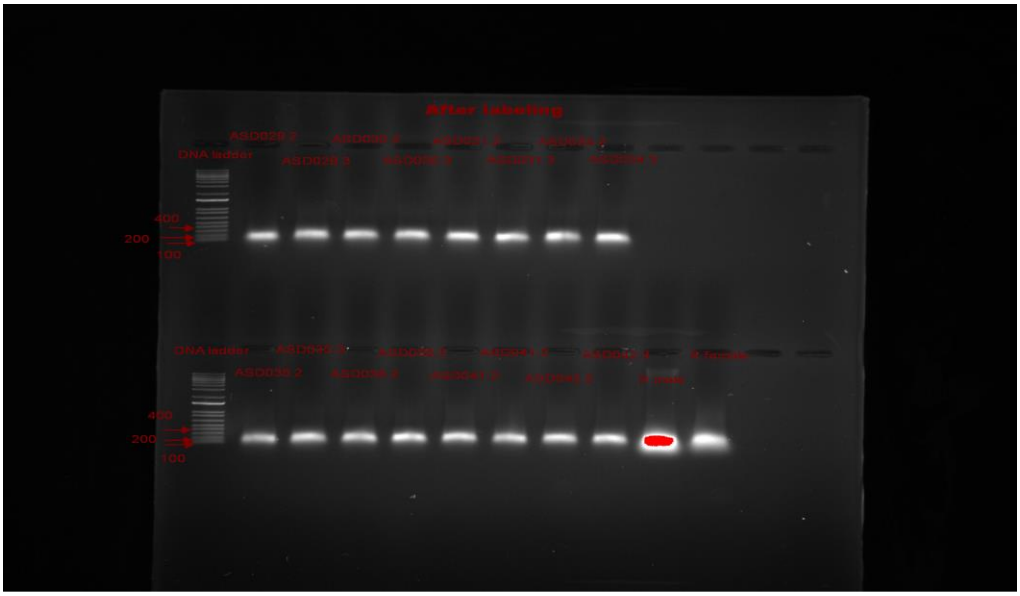


Supplementary figure 2: Comparation between normal brain and autism brain

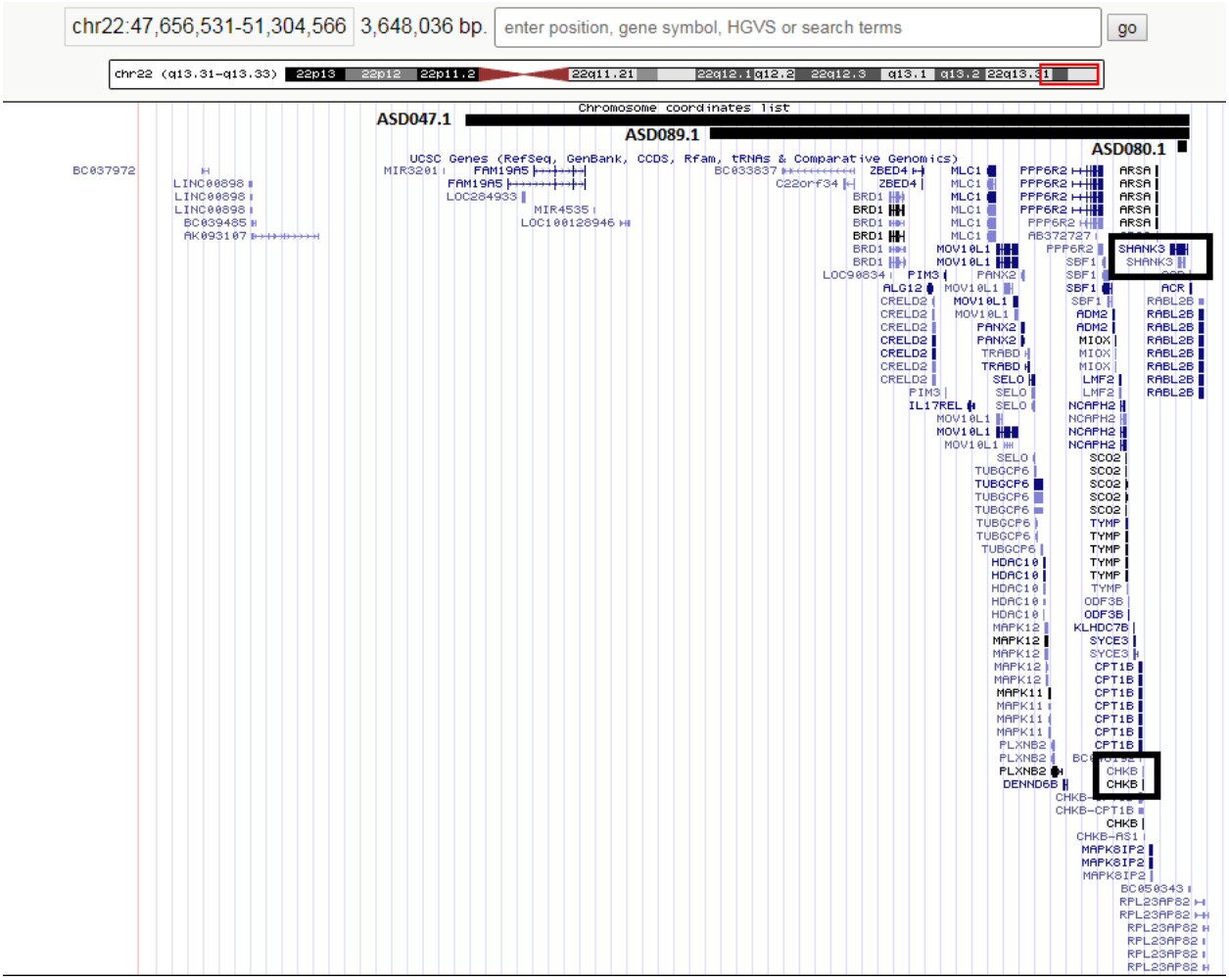
Representative normal image have Z-scores for most brain areas less than 2SD. Images shown have a 2 SD threshold applied. While Autism patient in both left and right brain shows green and yellow color that means 3-5SD.

A.



B. 

Supplementary figure 3: An example of gel electrophoresis after labeling DNA to check the effeciency of restriction steps in aCGH experiments A. Original DNA B. Labeling products



Supplementary figure 4: 3 patients (ASD047.1, ASD089.1 and ASD080.1) have a deletion CNV located in 22q13 with a part of *SHANK3* gene overlapping

The supplemental figure show CNVs have been found in 3 above patients with the containing gene list. These CNVs show the overlapping of a part of *SHANK3* gene

APPENDIX 3: MEDICAL RECORD

MEDICAL RECORD FOR AUTISM CHILDREN

Patient ID (sticker)

I	Administrative information		Notes
a1	Patient's full name	……	
a2	Gender	1. Male 2. Female	
a3	Date of birth	_ _/_ _/_ _ _ _ *(dd/mm/yyyy)*	
a4	Address	…… ……	
a5	Father name	……	
a6	Father date of birth	_ _/_ _/_ _ _ _ *(dd/mm/yyyy)*	
a7	Nation (father 3 generation)		
a8	Father occupation	1. State employee 2. Non-state employee 3. Freelance work 4. Farmer 5. Student 6. Househusband 7. Unemployed 8. Disabled 9. Refused to answer 10. Others (details): ……	

a9	Father academic level	1. Primary/Secondary school 2. High school 3. College 4. Undergraduate and Upper 5. Others (details): ……	
a10	Father mobile number	……	
a11	Father email address	……	
a12	Mother name	…….	
a13	Mother date of birth	_ _/_ _/_ _ _ _ *(dd/mm/yyyy)*	
a14	Nation (mother 3 generation)		
a15	Mother occupation	1. State employee 2. Non-state employee 3. Freelance work 4. Farmer 5. Student 6. Househusband 7. Unemployed 8. Disabled 9. Refused to answer 10. Others (details): ……	
a16	Mother academic level	1. Primary/Secondary school 2. High school 3. College	

		4. Undergraduate and Upper 5. Others (details): ……	
a17	Mother mobile number	……	
II	Reason for hospitalization		
a18	Reason for hospitalization	…… ……	
III	**Pathological process**		
a19	Pathological process details:	…… …… ……	
IV	**History of illness**		
b1	Paternal history of mental illness	1. Yes 2. No If yes, Please provide details: ..... What hospital gave diagnosis:	
b2	Paternal history of genetic diseases		
b3	Medicine (Deparkin, neuroleptic, addictive drugs,…):	Drug 1..... Dose..... ……	

		Using time before having this baby:.....(month) Drug 2..... Dose..... …… Using time before having this baby:.....(month) Drug 3..... Dose..... …… Using time before having this baby:.....(month)	
	Maternal history (including pregnancy history)		
b4	Maternal history of mental illness	1. Yes 2. No If yes, Please provide details: ..... What hospital gave diagnosis:	
b5	Maternal history of genetic diseases		
b6	Medicine (Deparkin, neuroleptic, addictive drugs,…):	Drug 1..... Dose..... ……	

		Using time before having this baby:.....(month) Drug 2..... Dose..... …… Using time before having this baby:.....(month) Drug 3..... Dose..... …… Using time before having this baby:.....(month)	
b7	Maternal pregnancy history	1. Diabetes 2. Hypertension 3. Genitourinary tract infection 4. Rubella infection 5. Viral infection 6. Rheumatoid 7. Stress 8. Other (details)…….	
b8	Threatening miscarriage or threatening premature	1. Yes 2. No	
b9	Complications of delivery	1. Preeclampsia 2. Eclampsia 3. Haemorrhage	

		4. Other (detail):……	
b10	Smoke exposure?	1. Yes 2. No If yes: 1. Direct 2. Indirect Amount: ……	
b11	Alcohol consumption?	1. Yes 2. No If yes, amount ……..ml/day	
b12	Pesticides/chemical exposure?	1. Yes 2. No	
b13	Types of pesticides or chemical	1. DDT, Duration:.....month 2. Diazinon (Agrozinon,...) Duration:.....month 3. Fenitronthion (Sumithion), Duration:.....month 4. Malathion, Duration:.....month 5. Pirimiphos methyl (Pirimicarb), Duration:.....month 6. Propagite (Acaricide), Duration:.....month 7. Other:....., Duration:.....month	

		8. Other:....., Duration:.....month	
b14	Frequency	1. Everyday 2. 1-2 times per week 3. 1-2 times per month 4. Other (Detail): …….	
	Maternal delivery history		
b15	Low amniotic fluid level	1. Yes 2. No	
b16	Delivery method	1. Vaginal delivery 2. Vacuum extraction or forceps delivery 3. Ceasarean	
b17	Duration of labor	……hours	
	History of this child		
b18	Order of this child in family	□ 1 □ 2 □ 3 □ 4 □ Other (details)…	
b19	Gestational week at birth	.....(weeks)	
b20	Weight at birth	……(gam)	
b21	Postnatal disease	1. Yes 2. No	

b22	**Disease**	**Duration of disease**	**Treatment**

	Treatment		
	Therapy	**Duration of treatment**	**Result**
b23	***Education:*** 1. Yes 2. No	_____ tháng	No improvement Little improvement Big improverment
b24	***Rehabilitation:*** 1. Yes 2. No	_____ months	No improvement Little improvement Big improverment
b25	***Drugs:*** 1. Yes 2. No	_____ months	No improvement Little improvement Big improverment
b26	***Stem cell transplantation:*** 1. Yes 2. No	_____ months	No improvement Little improvement Big improverment
b27	***Other therapies*** 1. Yes 2. No	_____ months	No improvement Little improvement Big improverment
b28	Family history of genetic autism spectrum disorder (ASD) (Especially mother, father or sibling with ASD)		
b29	Family history of mental diseases		
b30	Family history of genetic diseases		

V	Examination		Note
c1	General		
c2	Weight (kg)		
c3	High (cm)		
c4	Head circumference (cm)		
c5	Circulatory system	1. Normal 2. Abnormal	
c6	Respiratory system	1. Normal 2. Abnormal	
c7	Digestive system	1. Normal 2. Abnormal	
c8	Urinary system	1. Normal 2. Abnormal	
c9	**Criteria for mental illness**		
c9_1	DSM-V		
c9_2	CARS		
c9_3	ADOS		
c9_4	DENVER II		

	Tests		
c10	Test date	___ ___ / ___ ___ / ___ ___ ___ ___ (dd/mm/yyyy)	

c11	EEG	1. Normal wave 2. Epilepsy wave 3. Other abnormal wave	
c12	Progression of myelin by age	1. Normal 2. Abnormal	
c13	Congenital abnormality	1. Yes 2. No	
c14	Brain atrophy	1. Yes 2. No	
c15	Other damage	1. Yes 2. No	
	Other tests		
	Blood biochemical testes	GOT, GPT, Ure, Creatinin, Electrolytes, NH3, Lactate	
	Hematological tests:	CBC, blood group, blood coagulation	
	Microbiological tests:	HIV, HBsAg	
	Chest X-ray		
VI	**Diagnosis**		
f1	Autism level	1. Serve 2. Moderate 3. Mild	

Hanoi, Date ..... month ....year…

Doctor

APPENDIX 4: DIAGNOSIS TOOLS

AUTISM ASSESSMENT PER DSM – V (F84.0)

A. Persistent deficits in communication and social interaction across multiple contexts as manifested by the following 3 issues (currently or by history)

☐ Deficits in social-emotional reciprocity.

☐ Obvious deficits in nonverbal communicative behaviours for social interactions.

☐ Does not know how to develop, maintain and build close relationships.

B. Restricted, repetitive behaviours and interests, as manifested by at least 2 of the following (currently or by history).

☐ Stereotyped or repetitive movements, use of objects or speech.

☐ Inflexible adherence to routines or ritualized patterns (verbal or nonverbal behaviour).

☐ Restricted and fixated interests that are abnormal in intensity and focus.

☐ Hyper- or hyporeactivity or unusual interest in sensory aspects of the environment.

☐ **C. Symptoms must be present in the early developmental period (but may not become fully manifested until social demands exceed limited capacities)**

☐ **D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.**

☐ **E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism** spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and

intellectual disability, social communication should be below that expected for general developmental level**.**

CONCLUSION:

Note:

- Specify if: With/without intellectual impairment; With/without language impairment; Associated with a known medical condition, genetic condition or environmental factor; Associated with other disorders such as neurodevelopmental, mental and behavioral disorders.

- Autism disorder, Asperger, PDD- NOS are all classified under autism spectrum disorder (ASD)

- There are three levels of severity of ASD: Level 3: Requiring very substantial support; Level 2: Requiring substantial support; Level 1: Requiring support.

Table: Severity levels for autism spectrum disorder

Severity level	**Social communication**	**Restricted, repetitive behaviors**
Level 3 "Requiring very substantial support"	Level 2 "Requiring substantial support"	Level 1 "Requiring support"

Severity level	**Social communication**	**Restricted, repetitive behaviors**
Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and who has markedly odd nonverbal communication. Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful response to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to- and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.

Severity level	**Social communication**	**Restricted, repetitive behaviors**
	variety of contexts. Distress and/or difficulty changing focus or action.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence

CHILDHOOD AUTISM RATING SCALE (CARS)

Name of child:…….....Date of

birth:…….....Age(months):……...

Address:…….....Tel:…….....

Mother's

name:…….....Age:…….Occupation:…….....

Father's

name:…….....Age:…….Occupation:…….....

Assessed by:…… Date assessed:…….....

Serial	**Description**	**Score**						
1	Relating to people	1	1,5	2	2,5	3	3,5	4
2	Imitation	1	1,5	2	2,5	3	3,5	4
3	Emotional response	1	1,5	2	2,5	3	3,5	4
4	Body use	1	1,5	2	2,5	3	3,5	4
5	Object use in play	1	1,5	2	2,5	3	3,5	4
6	Adaptation to Change	1	1,5	2	2,5	3	3,5	4
7	Visual response (visual movement)	1	1,5	2	2,5	3	3,5	4
8	Listening response	1	1,5	2	2,5	3	3,5	4
9	Taste, smell & touch response	1	1,5	2	2,5	3	3,5	4
10	Fear and anxiety	1	1,5	2	2,5	3	3,5	4
11	Verbal communication	1	1,5	2	2,5	3	3,5	4
12	Nonverbal communication	1	1,5	2	2,5	3	3,5	4
13	Activity Level	1	1,5	2	2,5	3	3,5	4

14	Level and consistency of intellectual response	1	1,5	2	2,5	3	3,5	4
15	General Impression	1	1,5	2	2,5	3	3,5	4
Total								

Note: 15 - 30: Non-autistic; 30 - 37: Mild-moderate autism; 37 - 60: Severe autism.

In which: 37 - 40 points: Autism at Severity Level I;

40 - 45 points: Autism at Severity Level II; 45 points: Highly severe autism.