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Correlation of Major Iron Metabolism Related-Genes Single Nucleotide Polymorphisms (SNPs) with Autism Spectrum Disorder (ASD) in Palestinian Patients.

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Declaration

Here I declare that my MSc thesis entitled "Correlation of Major Iron Metabolism Related-Genes Single Nucleotide Polymorphisms (SNPs) with Autism Spectrum Disorder (ASD) in Palestinian Patients" is the result of my own research and was written independently with no other sources that quoted.

Sabha Fahmi Rabaya

Signature: Sabha Rabaya

Date: 18/7/2021

Dedication

I dedicate this work to my parents

Fahmi Rabaya and

Fawzeya Rabaya

Acknowledgments

I would like to acknowledge a number of people who made this research possible. Firstly, I would like to thank the participants and their families, for their time and effort. I wish to show my appreciation to my advisor Prof.Hisham Darwish for his guidance and support. I would also like to thank Miss.Sameera Nairat, Miss.Roua Thawabtah, and Mr.Hussam Sallam for their help and time.

Abstract

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders with genetic and environmental components. ASD diagnosis is currently based only on clinical criteria, which rises the need for early diagnostic genetic markers. As iron plays a crucial role in brain development, neurotransmitter synthesis, neurons myelination and mitochondrial function, we hypothesized that brain iron dysregulation may play a role in ASD development and pathogenesis. The aim of our study was to assess the association of a number of single nucleotide polymorphisms (SNPs) within transferrin receptor 1 (*TFRC*) rs11915082, solute carrier family 11 member 2 (*SLC11A2*) rs1048230 and rs224589, solute carrier family 40 member 1 (*SLC40A1*) rs1439816, and hepcidin antimicrobial peptide gene (*HAMP*) rs10421768 with ASD risk. The study included 48 Palestinian individuals with ASD and 88 non-ASD controls. Genotypes were determined by RFLP-PCR or Sanger's sequencing. Our data revealed that the rs11915082 G allele ($p=0.008$, $OR= 2.06$, 95% $CI=1.19-3.55$) and the rs1439816 C allele ($p<0.0001$, $OR=5.13$, 95% $CI=3.00-8.79$) were associated with ASD pathogenesis. At the genotype level, the polymorphisms rs11915082 ($p=0.041$), rs1439816 ($p=0.000$), and rs10421768 ($p=0.028$) showed a significant association with ASD overall phenotype. Our data demonstrate the role of iron dysregulation in ASD development and pathogenesis and indicate that some genetic variations in iron metabolism genes can be applied as genetic markers for early diagnosis of ASD.

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Abbreviations

ALS: Amyotrophic lateral Sclerosis
AS: Aspergers Syndrome
ASD: Autism Spectrum Disorder
BBB: Blood-Brain Barrier
BCSF: Blood-CerebroSpinal Fluid
CDC: Centre of Disease Control and Prevention
CI: Confidence Interval
CNV: Copy Number Variation
DCYTB: Duodenal Cytochrome B
DMT-1: Divalent Metal-ion Transporter1
DSM: Diagnostic and Statistical Manual of Mental Disorders
Fe(II): Ferrous iron
Fe(III): Ferric iron
FMRP: Fragile X Mental Retardation Protein
FPN1: Ferroportin
HAMP: Hepcidin Antimicrobial Peptide
HFE4: Hemochromatosis type 4
HH: Hereditary Hemochromatosis
HIF: Hypoxia-Inducible Factor
HRE: Hypoxia Response Element
HWE: Hardy–Weinberg Equilibrium
IRE: Iron Responsive Element
IRP: Iron Regulatory Protein
KCTD13: Potassium Channel Tetramerization Domain Containing 13
LfR: Lactoferrin Receptors
LfR-lf: Lactoferrin Receptor-Lactoferrin
MAP3: Mitogen Activated Protein Kinase 3
MeCP2: Methyl-CpG Binding Protein 2
NLGN3: Neuroligin 3
NLGN4: Neuroligin 4
Nramp-2: Natural resistance-associated macrophage protein
NTBI: Non-Transferrin Bound Iron
OR: Odds Ratio
PCR: Polymarase Chain Reaction
PDD-NOS: Pervasive Developmental Disorder Not Otherwise Specified
POGZ: Pogo Transposable Element Derived with ZNF Domain
RFLP: Restriction Fragment Length Polymorphism

ROS: Reactive Oxygen Species
SCN2A: Sodium Voltage-Gated Channel Alpha Subunit 2
SFARI: Simons Foundation Autism Research Initiative
SLC40A1: Solute Carrier Family 40 Member 1
SNP: Single Nucleotide Polymorphisms
SYN1: Synapsin-1
SYN2: Synapsin-1
Tf: Transferrin
TfR1: Transferrin Receptor 1
TfR2: Transferrin Receptor 2
UTR: Untranslated Region
WHO:World Health Organization

1. CHAPTER ONE: INTRODUCTION

1.1 Background

This chapter provides detailed information on the definition, history, diagnostic criteria, and etiology of Autism Spectrum Disorder (ASD). The role of iron is reviewed along with detailed description of its metabolism and homeostasis. Major iron metabolism related genes and their protein products are discussed along with description of specific single nucleotide polymorphisms in these genes.

1.1.1 History

The word “autism” comes from the combination of two Greek words, prefix “auto” which means “self” and the suffix “ism” meaning “the act or state of”. The word “autism” was used for the first time by the Swiss psychiatrist Eugen Bleuler in 1911 to describe the autistic thinking which was characterized by detachment from reality in cases with schizophrenia (Evans, 2013).

The use of the term “autism” has evolved over the years until taking its modern meaning and sense. In 1938, the Austrian pediatrician Hans Asperger published a paper in German in which he used the term “ autistic psychopathy” to describe autism within its modern sense (Asperger, 1938). Asperger’s adaptation of that term constructed the start point of the modern definition of autism. On the other hand, Leo Kanner, an American-Austrian child psychiatrist was the first to introduce the term “autism” in its modern meaning in English in the year of 1943.

Aside from the term “autism”, the disorder was probably first described in 1820s by Jean-

Marc-Gaspard Itard. As it was noticed by Carry, Itard described a group of symptoms within a group of cases under diagnosis with what he called “intellectual mutism” separating those cases from individuals diagnosed with mental retardation (Carrey, 1995). In the 1920s, a Russian neurologist named Grunja Jefimovn Ssucharewa described a group of symptoms in children, diagnosing the cases with “schizoid personality disorder” (Ssucharewa, 1926). Many decades later, it was recognized that she had described the main symptoms and hallmarks of autism (Ssucharewa & Wolff, 1996).

In 1943, Kanner published a paper entitled “Autistic Disturbances of Affective Contact” in which he discussed the condition of a group of eleven children who showed specific group of symptoms and behaviors such as, lack of social interaction and communication, language and speech delay, resistance to change, obsessive to sameness in their routines, and oversensitivity to stimuli (Kanner, 1943). Observations of Kanner became the basic criteria for the current understanding of ASD. One year after Kanner publication, in 1944, Hans Asperger published a paper in German in which he described the symptoms of a group of four boys at school-age and named their condition as “autistic psychopathy”. As was reviewed by Lyons & Fitzgerald, both Kanner’s and Asperger’s observations shared some similarity such as social deficits and unusual behaviors (Lyons & Fitzgerald, 2007). The world health organization (WHO) introduced autism officially for the first time in 1979 in the Ninth revision of the International Classification of Diseases (ICD-9) referring to it as infantile autism. In addition to that, Feinstein has documented that the WHO recognized the condition described by Asperger under the category “other specified pervasive developmental disorders” (Adam Feinstein, 2010). In turn, in the third edition of the American Psychiatric Association’s Diagnostic and Statistical Manual of Mental

Disorders (DSM-III) autism was recognized as a separate disorder from Schizophrenia under the category “pervasive developmental disorders” (Adam Feinstein, 2010).

In contrast to the previous published editions of the American Psychiatric Association’s DSM, the last published edition in May 2013, DSM-IV, followed a new approach in categorizing autistic conditions. DSM-IV introduced a new term “autism spectrum disorder“ under which all subcategories of pervasive developmental disorders lie with detailed severity levels (American Psychiatric Association., 2013).

1.1.2 Prevalence of ASD

A remarkable increase in the number of children diagnosed with autism spectrum disorder (ASD) within the last 20 years was observed globally. Fombonne documented in a paper published in 1999, that the general prevalence of autism was estimated to be about 5 in 10,000, with 4:1 male to female ratio (Fombonne, 1999). After the year 2000, the number of patients continued to rise up. Chakrabarti and Fombonne reported the prevalence of autistic disorder and all pervasive developmental disorders to be about 22 and 58 per 10,000 respectively (Chakrabarti & Fombonne, 2005). Studies conducted by the Centre of Disease Control and Prevention (CDC) in the United States of America showed an increasing trend of ASD prevalence from 90 per 10,000 in 8 years old children in 2006 to 113 per 10,000 in 2008 and 147 per 10,000 children in 2010 (CDC, 2009, 2012, 2014). Globally, data published in 2012 estimated that the median of ASD prevalence was 62 per 10,000 (Elsabbagh et al., 2012). Elsabbagh and colleagues also documented that there were no significant differences in ASD prevalence between populations in different geographic regions, and the data did not support the presence of strong impact of ethnic, cultural or economic factors on ASD prevalence (Elsabbagh et al., 2012). Several factors have been proposed to influence this rising trend of ASD prevalence including the improving and broadening of diagnosis criteria, earlier diagnosis, better service availability and increased awareness of ASD within both laypersons and professionals (Elsabbagh et al., 2012; Gernsbacher et al., 2005; Shattuck, 2006).

The last published data by CDC in the United States of America estimated the prevalence of ASD among 8 years aged children in 2016 to be about 185 per 10,000 (Maenner et al., 2020). On the other hand, the World Health Organization estimated the worldwide prevalence of ASD is about 62 per 10,000 (World Health Organization, 2019). One of the present hallmarks of ASD is the presence of sex differences in ASD prevalence with males being more diagnosed with ASD than females. Based on multiple studies conducted in the US and other countries, the estimated ratio of male to female ASD prevalence is 4:1(Fombonne, 2009; Idring et al., 2015; Werling & Geschwind, 2013).

Regarding the Arab world which consists of 22 nations located in North Africa and the Middle east with a whole population of more than 400 million with wide range of ethnic and cultural backgrounds (Hassan, 2019), available evidence estimates the prevalence of ASD in Oman is 1.2 per 10,000 children (Al-Farsi et al., 2012) 60 per 10,000 in Saudi Arabia (Aljarallah et al., 2007), and 59 per 10,000 in the United Arab Emirates (Eapen et al., 2007). Looking closely to the situation in Palestine, attention toward ASD has been increased in recent years, but there is a lack in ASD epidemiological studies which make the prevalence of ASD in Palestine unknown (Shawahna, 2017).

1.1.3 Autism spectrum disorder

ASD is a set of early onset neurodevelopmental disorders which all express a wide range of symptoms and difficulties in three major areas; social skills and interaction, communication skills, and behavioral flexibility (American Psychiatric Association., 2013). The early symptoms of ASD usually identified by the age of 1 to 3 years old (Newschaffer et al., 2007). ASD represent a group of disorders, including autism, the most severe one, Asperger's Syndrome (AS), and pervasive developmental disorder not otherwise specified (PDD-NOS) (Levy et al., 2009; Szpir, 2006).

1.1.3.1 ASD symptoms and diagnosis

ASD is characterized by a wide range of symptoms which mainly include, lack of social interaction, lack of verbal and nonverbal communication and the presence of stereotyped and repetitive behaviors, activities and interests. Despite the fact that individuals diagnosed with ASD have impairments in the previously mentioned core areas, it is obvious that the clinical phenotype, severity and symptoms frequency within ASD patients are very heterogeneous (Lenroot & Yeung, 2013). It has been observed that the pattern of ASD symptoms changes during the lifetime of an autistic individual. While language deficits and hyperactivity behaviors are common symptoms in most autistic children during their early childhood, relationships and mood regulation problems appear during their adolescence (Nazeer & Ghaziuddin, 2012).

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has represented a new, more useful and accurate criteria of autism-related disorders. To meet the DSM-5 criteria of ASD diagnosis, individuals with ASD must show symptoms from early childhood, which include, ‘persistent deficits in social communication and social interaction’ and ‘restricted, repetitive behavior, interests or activities’(American Psychiatric Association., 2013). DSM-5 criteria with examples are summarized in Box1.

In addition to the core diagnostic features of ASD, great attention has been focused on other non-diagnostic features over the past decade including, gastrointestinal problems (Samsam et al., 2014), sleep disorders (Karthikeyan et al., 2020), immune system problems (Hollis et al., 2017), and oxidative stress (Manivasagam et al., 2020).

Box 1. DSM-5 Summary of ASD diagnosis Criteria

A. Persistent impairments in social communication and interaction , include

1) difficulties in social initiation and response such as
    - failure of back and forth conversation
    - reduced sharing of interests and emotions
    - poor social imitation
2) problems with nonverbal communication such as
    - poor eye contact
    - Impairment in the understanding and use of body postures
    - Impairment in the understanding and use of gestures (e.g. pointing, waving)
3) difficulties with social awareness and social relationships such as
    - Difficulties in making and maintaining relationships (inability to take another person’s perspective).
    - Deficits in adjusting actions and behaviors to suit contexts (e.g. failure of noticing another person’s interest or lack of interest).
    - Difficulties in making friends and lack of interest in others.

B. Restricted or repetitive patterns of interests, behaviors or activities, at least presented with 2 of the following 4 symptoms

1) Stereotyped or repetitive speech, use of subjects or motor movements
    - Stereotyped or repetitive speech such as
        - Echolalia
        - Refers to self by own name
        - Repetitive vocalizations
    - Stereotyped or repetitive use of objects such as
        - Nonfunctional play with objects
        - Repetitive opening and closing of things(e.g. doors)
    - Stereotyped or repetitive motor movements such as
        - Repetitive hand movements
        - Excessive teeth grinding

2) Adherence to routines, resistance to change, or ritualized patterns of verbal or nonverbal behavior. Examples
    - Insistence on same food
    - Difficulties with transitions
    - Repetitive questioning
3) Exceedingly restricted, fixated interests that are considered abnormal in intensity or focus. Examples
    - Focused on the same objects, topics or activities
    - Attachment to unusual objects
    - Unusual fears
4) Hyper-or hypo-reactivity to sensory input or unusual interest in sensory aspects of environment. Examples
    - High tolerance for pain
    - Unusual visual exploration or activity (e.g. Extreme interest with watching movement of things)
    - Odd responses to sensory input
    - Licking or sniffing objects

C. Symptoms must be present in early childhood.

D. Symptoms cause limitation and impairment of everyday functioning.

1.1.3.2 ASD etiology and risk factors

The causes of ASD are still poorly understood. Currently ASD is considered a multi-factorial neurodevelopmental disorder caused by genetic and non-genetic (environmental) factors and their interaction.

1.1.3.2.1 Environmental factors

Accumulating evidence has shown a significant role of environmental factors in the pathogenesis of ASD (Hallmayer et al., 2011; Lyall et al., 2014). It was documented that exposure to environmental factors may affect brain development and different neurological processes such as cell differentiation, axon myelination and synaptogenesis (Lyall et al., 2014).

A comprehensive meta-analysis have been conducted by Gardener and colleagues to examine the association of a group of more than 60 prenatal and neonatal factors with autism risk (Gardener et al., 2011). In that study, factors linked with autism risk included birth injury or trauma, umbilical-cord complications, abnormal presentation, fetal distress, maternal hemorrhage, multiple birth, summer birth, low birth weight, small gestational age, low 5-minute Apgar score, congenital malformation, feeding difficulties, meconium aspiration, neonatal anemia, ABO or Rh-incompatibility and hyperbilirubinemia. On the other hand and in the same study, factors not associated with autism risk were assisted vaginal delivery, anesthesia, postterm birth, high birth weight, and head circumference (Gardener et al., 2011).

A genome-wide sequencing study showed that paternal age is associated with increasing risk of autism (Kong et al., 2012). Advanced paternal age was shown to be associated with an increasing rate of de novo mutations, that increase the risk of ASD and schizophrenia (Kong et al., 2012). Many maternal environmental factors have been linked to autism risk during pregnancy, which included valproic acid, thalidomide, alcohol, cytomegalovirus, rubella, depression, schizophrenia, obsessive-compulsive disorder, autoimmune disease, stress, allergic reaction, and hypothyroidism (King, 2011). These factors may affect brain development, initiate the expression of retinoid acid and/or estradiol sensitive genes whether by directly increasing or reducing the production of human alpha-fetoprotein (King, 2011). Pre-conceptual exposure to many environmental mutagens has been linked to autism risk by causing de novo mutations. Mercury, cadmium, nickel, trichloroethylene and vinyl chloride have been suggested to increase the risk of autism (Kinney et al., 2010). Exposure to traffic-related air pollution (Particulate Matter) during gestation and the first year of life has also been shown to contribute to autism (Volk et al., 2013).

Prenatal vitamin D deficiency has shown to increase the risk of autism, as it affects brain development and function (Eyles et al., 2013). Moreover, children with ASD have been reported to have significantly low levels of vitamin D in their plasma in comparison to controls (Eyles et al., 2013). Residence in regions that are urbanized, located in higher latitudes, or experience high levels of precipitation has been linked to increased autism risk as it is associated with decreased sun exposure and increased risk for vitamin D deficiency (Kinney et al., 2010).Vitamin D has essential roles in DNA damage repair and protection against oxidative stress hence, vitamin D deficiency result in higher mutation rates and impairments in DNA repair (Kinney et al., 2010).

Overall, many environmental factors have shown to contribute widely to autism. It is worth mentioning here that no single environmental factor is sufficient enough to profoundly affect the predisposition to ASD, a high effect would be caused by a combination of several environmental factor (Gardener et al., 2009)

1.1.3.2.2 Genetic factors

Since the beginnings of research on ASD, it was initially assumed to be of environmental origin. After the observations that autism incidence among siblings was 50x higher than average, the role of genetics was suggested. Twin studies have documented that monozygotic twins were more likely to share the diagnosis than dizygotic twins (Folstein

& Rutter, 1977). Early karyotype studies documented chromosomal abnormalities (Gillberg & Wahlström, 1985), but as a result of the low resolution of these studies it was impossible to determine specific genes correlated with autism conditions. Candidate genes sequencing was used to identify genes in loci with frequent deletions and/or duplications in autism. Many genes have been identified using this approach such as methyl-CpG binding protein 2 (*MeCP2*) which is essential for neurons maturation, neuroligin 3 (*NLGN3*), and neuroligin 4 (*NLGN4*) (Carney et al., 2003; Jamain et al., 2003). Neuroligins are proteins important for cell-to-cell interactions between neurons (Amberger et al., 2015).

With the advent of high throughput sequencing, ASD research has improved, and ASD was confirmed to be multigenic and highly heterogeneous with the average case being a product of many susceptibility-increasing variations. Large-scale genetic studies on ASD patients and their families has identified hundreds of risk gene, the majority of these genes have shown to come from two broad classes. The first group includes genes involved in synapse formation, such as genes encoding ion transport proteins such as sodium voltage-gated channel alpha subunit 2 (*SCN2A*) and synaptic Ras GTPase activating protein 1 (*SYNGAP1*) (Stessman et al., 2017). Another type of genes included in the first group are genes encoding synaptic vesicle cycling proteins synapsin-1 (*SYN1*) and synapsin-2 (*SYN2*) (Giovedí et al., 2014). The second group of ASD risk genes involves genes encoding factors involved in transcriptional regulation and chromatin remodeling pathways such as pogo transposable element derived with ZNF domain (*POGZ*) and fragile X mental retardation protein (*FMRP*) (Stessman et al., 2017; Tran et al., 2019).

Copy number variations have been documented in ASD patients, and proved to be important contributing factor in ASD susceptibility, with estimation of causing roughly

10% of ASD cases (Geschwind, 2011). An example of investigated structural variants is 16p11.2 duplications, this region includes 25 genes, with the majority of them being highly active during the development of the nervous system and are critical for proper formation (Blaker-Lee et al., 2012). Golzio and his colleagues reported that only one gene in this region, potassium channel tetramerization domain containing 13 (*KCTD13*), is likely to be the key driver for the neurodevelopmental phenotypes associated with the 16p11.2 CNV (Golzio et al., 2012). Another gene in the 16p11.2 region, mitogen activated protein kinase 3 (*MAP3*) was suggested to be a driver of disease by studies on mice models (Pucilowska et al., 2015). Overall, it is likely that the impact of 16p11.2 duplications or deletions on neurodevelopmental disorder is not from just one gene, but from different genetic interactions (Iyer et al., 2018).

Taking everything into consideration, ASD is now understood to be a disorder of complex interactions between both genetic and environment factors (Chaste & Leboyer, 2012).

1.1.4 Iron

Iron is a vital metal in all living organisms (Nadadur et al., 2008). The ability of iron to easily lose or gain electrons make it an important participant in a variety of essential cellular oxidation and reduction reactions. Iron is also a cofactor for a lot of enzyme with essential roles within the human body. It is also vital for oxygen transport and delivery, since it is the cofactor for heme, which is a necessary component of hemoglobin, the molecular responsible for oxygen transport (Mackenzie et al., 2008).

Except epithelial cells shedding (Leecharoenkiat et al., 2016) and menstruation, there is no known iron excretion system. Thus, restricted amounts of iron should be present in human for keeping good health status. Excess iron (iron overload) or reduced iron (iron deficiency) is pathologic to human body and both have deleterious effect on the survival of cells (Nadadur et al., 2008). Normal adult human has about 3-4 g of iron (Fuqua et al., 2012) and about 50% of iron content is used in hemoglobin synthesis, while the rest is used for myoglobin and other iron-containing protein synthesis (Nadadur et al., 2008). Iron absorption, transport, uptake, metabolism, storage and recycling are controlled by highly regulated processes and mechanisms in which a lot of proteins play critical roles in each step. At the molecular level, mutations and variations in several proteins involved in iron homeostasis can have different effects on iron balance and homeostasis in human (Nadadur et al., 2008). Iron homeostasis can be studied through its use mainly by four types of cells, enterocytes in the small intestines which are responsible for intestinal iron uptake, erythrocyte precursors in the bone marrow in which iron is involved in hemoglobin synthesis, macrophages in spleen, liver and bone marrow which recycle iron from red blood cells destruction, and hepatocytes where iron is stored (Knutson, 2017; Yiannikourides & Latunde-Dada, 2019).

1.1.4.1 Overview of iron homeostasis

The major source of iron is from diet, and comes in many forms mainly categorized as heme iron and non heme iron and each form has a different absorption pathway (Fuqua et al., 2012). Figure (1.1) provides general summary of iron metabolism (Cronin et al., 2019). Non-heme dietary iron mostly found in the oxidized state Fe (III) (ferric iron) that has low bioavailability and solubility. For being intestinally absorbed ferric iron should be reduced to the ferrous form Fe(II) mostly be duodenal cytochrome B (DCYTB) (Nadadur et al., 2008). Intestinal iron absorption is a major step in maintaining balanced levels of iron within the body. It takes place mostly in the proximal small intestine, the duodenum and proximal jejunum, where iron crosses enterocytes by an Fe(III) iron transporter, located on the apical surface of enterocytes, called divalent metal-ion transporter1 (DMT-1) (Fuqua et al., 2012; Nadadur et al., 2008), also known as natural resistance-associated macrophage protein Nramp-2 (Diego, 1998). Intestinal iron absorption is a very regulated process by many mechanisms to provide the body with sufficient amounts of iron (Steele et al., 2005), and at the same time be quickly responsive for either increasing body requirement of iron or in situations where excess iron is present in the body to reduce the amount of absorbed iron since no mechanism is known for iron excretion. A major regulatory mechanism of intestinal iron absorption is controlled by the hormone hepcidin which is expressed by hepatocytes. In normal individuals, when iron levels increase, hepcidin reduce iron release from cells including enterocytes through binding with the only known iron export transmembrane protein in mammals called ferroportin (FPN1) (Fuqua et al., 2012). This binding results in ferroportin phosphorylation by Jak2 kinase which result in ferroportin internalization and degradation (Mariani, et al., 2009).

Following iron intake by enterocytes, it is either stored or transported by the transmembrane iron exporter located on the basolateral of enterocytes, differentiating erythrocytes and hepatocytes (Leecharoenkiat et al., 2016), ferroportin (FPN1), into the blood circulation to be transported to the various body tissues for utilization (Fuqua et al., 2012). Ferroportin can only transport iron in the ferrous form Fe(II) (Mitchell et al., 2014). Iron transported into circulation becomes predominantly bound to an iron carrier protein called transferrin that can only bind to ferric iron Fe(III), therefore a part of intestinal export of iron by ferroportin is being oxidized to ferric. Hephaestin is a membrane bound ferroxidase that is responsible for the oxidation of enterocyte released ferrous iron to ferric form (Knutson, 2017) .

Cellular iron uptake takes place mainly via transferrin receptor mediated endocytosis. Two transferrin receptors are found on the surface of different cells, transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2). TfR1 has a higher binding affinity to transferrin bound iron, thus expressed in the majority of cells, while TfR2 is found mainly in hepatocytes (Nadadur et al., 2008). TfR1 has about 25 fold increased transferrin bound iron binding affinity compared to TfR2 (Graham et al., 2008).

Transferrin bound iron first binds to the transferrin receptor at the cell surface forming iron-transferrin receptor complexes which enter the cell through clathrin-mediated endocytosis (Nadadur et al., 2008). Inside the cell, iron-transferrin receptor complexes trafficked to the early endosomes and acidification of these complexes results in iron release and then iron is converted to the ferrous form. Endosomal ferrous iron is then transported into cytosol by endosomal DMT1, followed by transferrin recycling, in which apo-transferrin dissociates from TfR1 and is used again for iron transport and delivery (Nadadur et al., 2008).

A percent of iron stored in the body is bound to ferritin and hemosiderin in different tissues within the body including liver, spleen, duodenum and other cell types (Saito, 2014). Ferritin which is the major storage form has a storage capacity of about 4500 ferric iron(III) ions (Nadadur et al., 2008). Another form of stored iron is the form of water insoluble protein, hemosiderin (Nadadur et al., 2008). Iron recycling is an important part of iron homeostasis, Macrophages in the spleen and liver are responsible for the destruction of senescent and damaged erythrocytes (Nadadur et al., 2008).

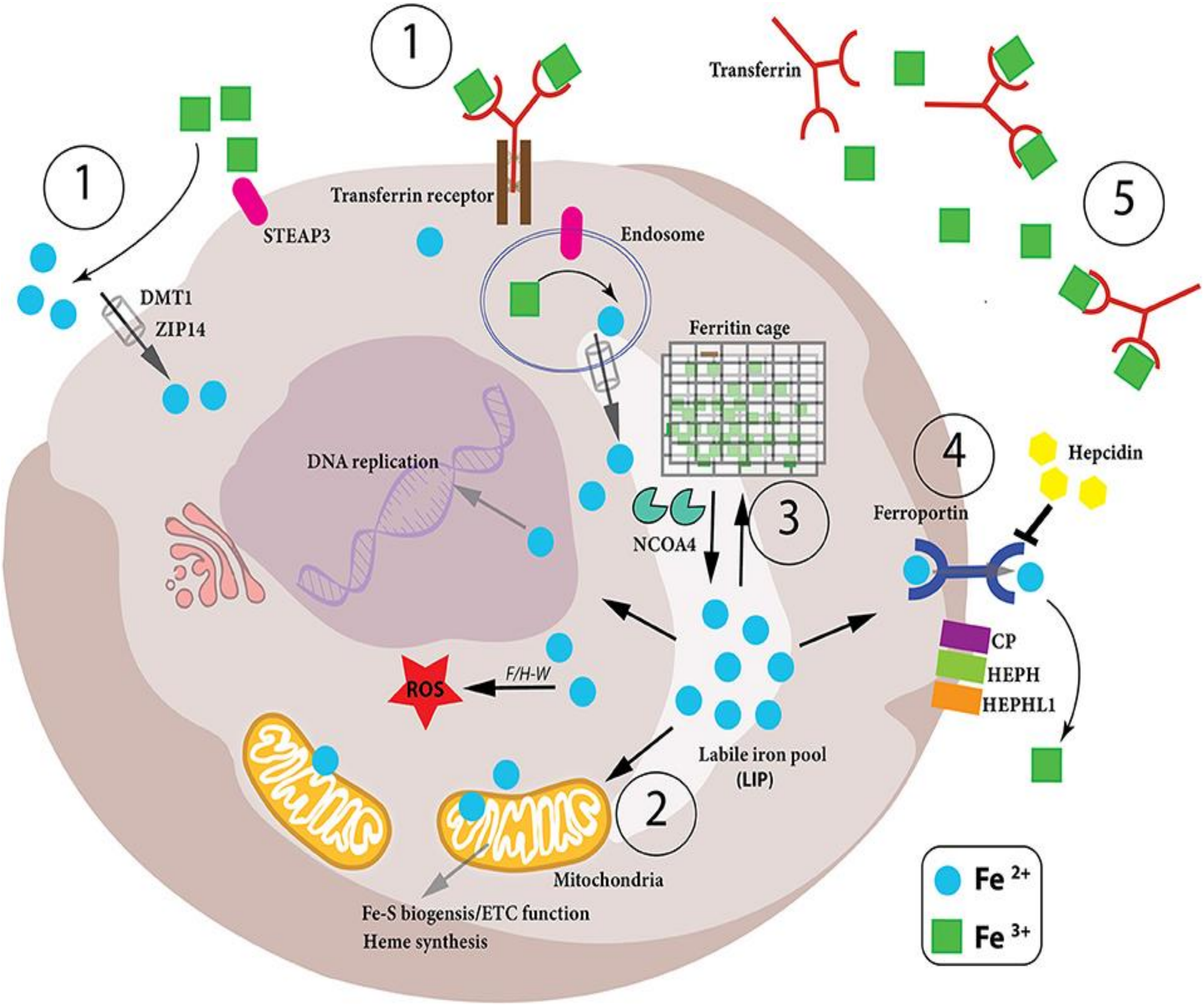

Figure 1.1. Cellular iron metabolism. Iron metabolism mainly includes (1) iron uptake,(2) iron utilization,(3)iron storage,(4)iron export, and (5) iron circulation. (Cronin et al., 2019).

1.1.4.2 Brain iron homeostasis

For iron to enter the brain, it needs to pass two barriers, the blood-brain barrier (BBB) and BCSF (blood-cerebrospinal fluid) (Pardridge et al., 1987). Figure (1.2) summarizes brain iron homeostasis (Ward et al., 2014). Transferrin bound iron enters the endothelial cells of the BBB through the transferrin receptors (TfRs) by endocytosis; TfR1 is expressed in high amounts on the luminal side of endothelial cells (Jefferies et al., 1984). Iron can also enter endothelial cells of the BBB as low molecular weight complex or independently as non-transferrin-bound iron. Then Fe is translocated across the endosomal membrane through the divalent metal transporter1 (DMT1) (Crichton, 2016). This Tf-TfR pathway is the most common pathway by which Fe crosses the BBB (Batista-Nascimento et al., 2012). Another suggested pathway is the lactoferrin receptor-lactoferrin (LfR-lf) pathway (Batista-Nascimento et al., 2012). Fe containing lactoferrin binds to lactoferrin receptors (LfR) resulting in Fe entering the cell and it then binds to ferritin and accumulates around the neuromelanin (Batista-Nascimento et al., 2012).

Iron is exported at the abluminal membrane of endothelial cells by unknown pathways (Ward et al., 2014). One of these pathways may involve ferroportin, by which Fe(II) is released at the basolateral surface of endothelial cells and oxidized to Fe(III) by ceruloplasmin (Jeong & David, 2003; Singh et al., 2014). Released iron binds to transferrin in the brain interstitial fluid and CSF (Singh et al., 2014). Brain Tf is synthesized by cells of the choroid plexus and oligodendrocytes (Crichton, 2016).

Different cell types in the brain acquire iron by different pathways. Neurons, acquire most of their needs of iron from Tf using the Tf-TfR system as neurons express abundant TfR levels (Crichton, 2016). Neurons export iron likely by FPN (Boserup et al., 2011). On the other hand, astrocytes do not express TfR, but express DMT1, so astrocytes probably take up iron by DMT1 (Crichton, 2016; Lane et al., 2010; Singh et al., 2014). Astrocytes iron efflux mostly carried out through FPN and glycophosphatidylinositol-anchored Cp (Jeong & David, 2003;Wu et al., 2004).

1.1.4.3 Iron and brain development, cognitive performance and behavior

Iron is essential for a group of vital functions during development, it should be present in adequate amounts for the child to develop healthy. Brain development is highly sensitive to environmental factors. One important factor is nutrients deficiency. The prenatal period and first three years of child life are very critical time for neurodevelopment, and high rates of essential neuro processes such as neurogenesis, myelination, and synaptogenesis occur simultaneously, resulting in tremendous brain growth (Tierney & Nelson, 2009). Iron functions and importance for brain development and function have been widely studied and investigated (Beard et al., 1993). Iron is distributed with different concentrations in different regions of the brain (Beard & Connor, 2003). Oligodendrocytes contain the highest concentrations of brain iron (Ward et al., 2014).Therefore, iron is essential for the enzymes involved in the myelination process and for monoamine neurotransmitter synthesis (Lozoff, 2007). In the CNS, iron-containing enzymes and proteins are involved in essential processes including oxygen transportation, DNA synthesis, myelin synthesis, mitochondrial respiration and neurotransmitter synthesis and metabolism (Crichton, 2016). All these facts suggest that iron metabolism may have important role in ASD pathophysiology.

Many studies have investigated the effect of iron deficiency on brain development. Iron deficiency has found to affect the formation of myelin sheaths resulting in impairments in

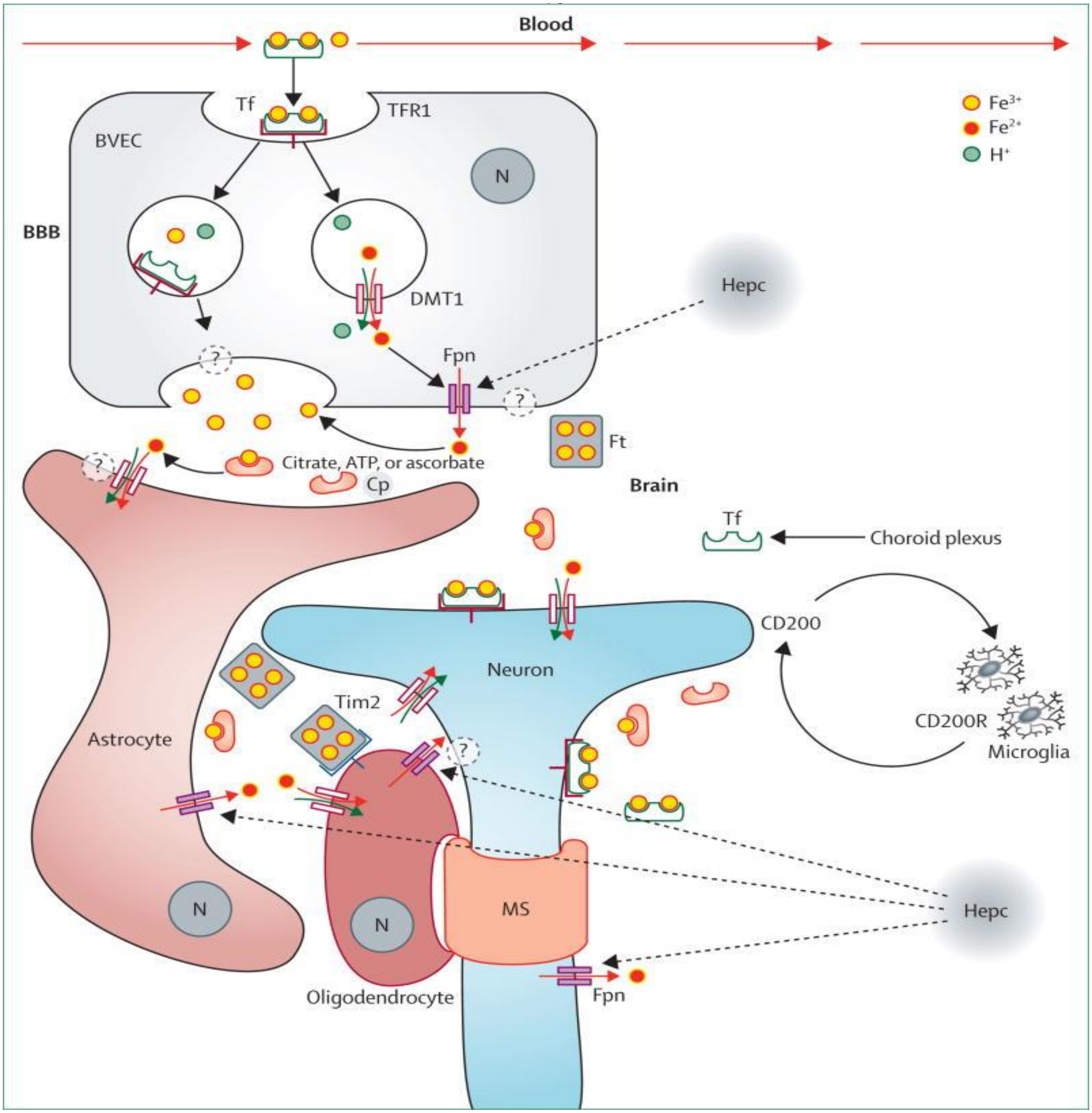

Figure 1.2. Iron homeostasis in the brain. Iron should cross the blood brain barrier. Iron uptake by endothelial cells of BBB take place mainly by TfR1. As iron is released from the abluminal side of the endothelial cells of the BBB it can bind to transferrin or to low molecular weight molecules (e.g. ascorbate, citrate, or ATP) to form nontransferrin-bound iron. cells of the brain such as Astrocytes and neuron can uptake and utilize iron via different mechanisms (Ward et al., 2014).

Animal studies showed that iron deficiency can alter neuronal metabolism and growth of dendrites which result in impairments of brain cell structure and function (Jorgenson et al., 2003; Rao et al., 2003). In humans, iron deficiency has been found to cause neural developmental impairments that result in impairments in learning, memory, attention, and psychomotor processes (Lozoff, 2007). Poor writing, reading, motor function, school progress, and more anxiety, depression and social problems had been experienced by children who had iron deficiency during infancy (n=48) in comparison with children who had normal iron levels as infants (n=14) (Lozoff et al., 2000).

In contrast to iron deficiency, iron accumulation and dysregulation of its metabolism my also contribute to neurological diseases. Nonbound iron is considered harmful mainly due to its redox activities, where abnormal iron homeostasis might cause cellular death or severe cellular dysfunction (Salvador et al., 2010). Many neurological disorders have been reported to be triggered by iron metabolism dysregulation. These include Alzheimer’s disease (Liu et al., 2006), Parkinson’s disease, Huntington’s disease, and Amyotrophic lateral Sclerosis (ALS) (Youdim et al., 2005). Iron ability to donate and accept electrons results in its ability to lead to the formation of reactive oxygen and nitrogen species (ROS). ROS result in oxidative damage of proteins, lipids and nucleic acids and contribute to cellular dysfunction and death which in turn can result in many disorders (Finkel & Holbrook, 2000).

Iron mediated oxidative damage has been thought to contribute to ASD pathogenesis (Manivasagam et al., 2020). As a result of its limited antioxidant capacity, high energy requirements and high levels of unsaturated lipids and iron, it has been proposed that oxidative stress in developing brains contributes to neural damage in genetically susceptible children (Chauhan et al., 2012). Brain accounts for only 2% of body mass but consumes 20% of oxygen (Andreone et al., 2015). Children are more vulnerable to oxidative stress than adult due to low Glutathione (GSH) levels (Erden-Inal et al., 2002). Accumulating evidence suggests the role of oxidative stress in the pathophysiology of ASD since levels of oxidative stress markers for protein oxidation, lipid peroxidation, and DNA oxidation are increased in autistic patient's blood (Chauhan & Chauhan, 2006; Chauhan et al., 2004; Chen et al., 2021; Meguid et al., 2011; Zoroglu et al., 2004), urine(Ming et al., 2005) and brains (Chauhan et al., 2011; Evans et al., 2008; López-Hurtado, E. & Prieto, 2008; Sajdel-Sulkowska et al., 2009) in comparison to control subjects. In addition to that, decreased activities of antioxidant enzymes and levels of antioxidant proteins such as transferrin and ceruloplasmin have been identified in blood samples from autistic subjects (Chauhan et al., 2004; Meguid et al., 2011; Yorbik et al., 2002; Zoroglu et al., 2004).

Restrictive or selective diet patterns, unusual feeding patterns, and unusual behaviors regarding food and meal times are common in children with ASD. Restrictive diet patterns for a prolonged period of time expose the autistic child to a potential health risk due to the development of nutritional deficiencies (Cermak et al., 2010). Regarding iron status, various studies have found autistic children to be more at risk of having inadequate iron levels (Keen, 2008) and high prevalence of iron deficiency has been documented within autistic children (Hergüner et al., 2012). A Turkish study found that 24.1% of 3-16 year old autistic children had iron deficiency and 15.5% had iron deficiency anemia (Hergüner et al., 2012). A study held on a group of Qatari autistic children to investigate iron deficiency anemia and Vitamin D deficiency among autism children found that iron deficiency, iron deficiency anemia and Vitamin D deficiency were more common in autistic subjects compared to control children (Bener et al., 2017).

According to the Simons Foundation Autism Research Initiative (SFARI) gene database, genetic variations in three different genes related to iron metabolism and homeostasis have been reported to be associated with ASD, including Alkylglycerol Monooxygen (*AGMO*), a gene encoding an iron dependent enzyme, Frataxin (*FXN*) a gene encoding a protein involved in mitochondrial iron transport, and Solute Carrier Family 25 Member 39 (*SLC25A39*) which encodes a protein involved in iron homeostasis.

1.1.5 Genes of interest

With the improvement of different DNA sequencing and mapping techniques, recent years have seen a huge increase in genetic studies identifying single nucleotide polymorphisms (SNPs) correlated with increased or decreased risk of pathogenesis of a variety of disorders. Several SNPs have been identified in key genes involved in iron metabolism with potential correlation with neuronal disorders. This research studied the possible correlation of specific SNPs in the below iron metabolism related genes with ASD. The correlation of these SNPs with the development of ASD provide a tool for more understanding of the pathogenesis of ASD and may provide a tool for early confirmatory diagnosis.

1.1.5.1 Transferrin Receptor (*TFRC*) gene.

TFRC gene is located on chromosome 3q29 as shown in figure(1.3 A) (Kent et al., 2002). *TFRC* encodes for a cell surface receptor TfR1, a glycoprotein with a molecular weight of about 90 kDa expressed as a homodimer in the majority of cells (Nadadur et al., 2008). It is expressed at low levels in most tissues, with higher levels in the placenta, activated lymphocytes, erythroid precursors, osteoclasts, microglia, intestinal crypt cells, and many types of cancers (Wu et al., 2016). TfR1 is essential for iron uptake by erythrocytes and other cell types by mediated endocytosis. It has high binding affinity to transferrin bound iron (Nadadur et al., 2008). Holo-Tf binds to TfR1 on the cell surface (Cheng et al., 2004), which initiates internalization of the complex by cathrin-mediated endocytosis (Lawrence et al., 1999). TfR1 and Tf binding is dependent on pH; iron saturated holo-Tf binds to TfR1 at pH 7.4 but iron-free apo-Tf cannot bind to TfR1 at this pH. On the other hand, at lower endosomal pH, TfR1 binds to apo-Tf but not to holo-Tf (Kawabata et al., 2000; Wally et al., 2006). TfR1 expression is regulated at both transcriptional and post-transcriptional levels. In situations of hypoxia or iron deficiency, the expression of hypoxia-inducible factors (HIF-alpha and HIF –beta) increases (Xu et al., 2017). These factors bind to the hypoxia response element (HRE) in the promoter of *TFRC*, resulting in increased *TFRC* expression (Xu et al., 2017). At the post-transcriptional level, the iron regulatory protein (IRP)-iron responsive element (IRE) system has an important function (Rouault, 2006). Five IREs are located in the 3'-untranslated region (UTR) of the *TFRC* mRNA, in the presence of intracellular iron deficiency, IRPs binds to IREs stabilizing *TFRC* mRNA which enhance TfR1 expression (Walden et al., 2006; Yikilmaz et al., 2005). In contrast, with excess iron, IRPs lose their interaction with IREs, resulting in TfR1 mRNA destabilization and degradation.

Reduced *Tfrc* expression is associated with anemia in mice (Zhu et al., 2008). Decreased *TFRC* expression was also documented in neuromelanin cells in restless legs syndrome (Connor et al., 2004). It was also documented that transferrin receptor densities were significantly decreased in the hippocampus and the temporal and occipital cortex but were unchanged in the frontal and parietal cortex and the cerebellum in patients with Alzheimer's disease (Kalaria et al., 1992). It was also suggested that increased plasma TfR1 levels might be a trait phenomenon in bipolar disorder (Tsai et al., 2003). As *TFRC* has been linked with iron dysregulation in many pathological conditions, this research investigated the association between ASD pathogenesis and the genotype of *TFRC* gene polymorphism: rs11915082 (c.-253G>A) that is located in the 5'-upstream region of the *TFRC* gene as shown in figure (1.3 B) (Kent et al., 2002). This SNP has been linked to age-related macular degeneration risk (Wysokinski et al., 2015). Figure (1.3 C) shows transcripts of *TFRC* gene (Kent et al., 2002).

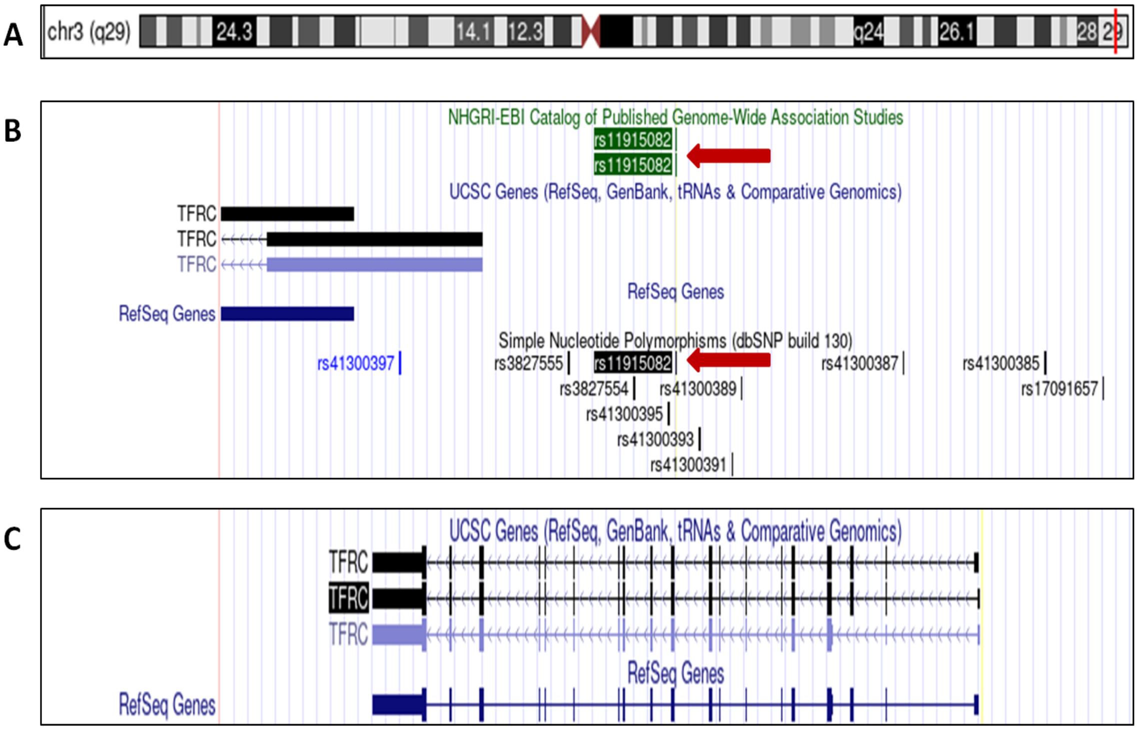

Figure 1.3. *TFRC* gene. A. location of *TFRC* gene on chr3, B. location of rs11915082 SNP in *TFRC* gene and C. *TFRC* transcripts (Kent et al., 2002)

1.1.5.2 Solute Carrier Family 11 Member 2 (*SLC11A2*) gene

SLC11A2 is located on chromosome 12q13 as shown in figure (1.4 A) (Kent et al., 2002). It encodes for DMT1, a ferrous iron transporter located on the surface of enterocytes in the small intestines, and responsible for the import of iron across the enterocytes (Nadadur et al., 2008). This protein also mediate iron transport across endosomes to the cytosol (Nadadur et al., 2008). In addition to its affinity for iron, DMT1 has an affinity for other cations such as cadmium, copper, cobalt, manganese, and zinc (Garrick et al., 2006; Sankaranarayanan et al., 2019). Conservation analysis shows that DMT1 is highly conserved within species. A DMT1 missense mutation (G185R) in Belgrade rats with autosomal inherited microcytic hypochromic anemia, found to cause disruption of iron transport, which indicates that DMT1 is vital for iron homeostasis (iron intestinal uptake and iron transport) (Diego, 1998). Deletion of *SLC11A2* in mice resulted in reduced enterocyte iron uptake (Gunshin et al., 2005). In humans, mutations or polymorphisms of *SLC11A2* gene suggested to have effect on human health through metal traffic disruption or metal ions overload (Gunshin et al., 1997; Kayaaltı et al., 2015; Lee et al., 1998). Different polymorphisms in *SLC11A2* gene have been documented to be associated with many disorders including, age-related macular degeneration (Wysokinski et al., 2012), Alzheimer's disease (Jamieson et al., 2005), microcytic anemia (Kloss-Brandstätter et al., 2012; Mims et al., 2005), Parkinson's disease (He et al., 2011), and Wilson's disease (Przybyłkowski et al., 2014). In line with the mentioned association of DMT1 with several conditions, this research investigated the association of two polymorphism located in this gene. Rs224589 which as an intronic SNP, figure (1.4 B) and rs1048230, which is located in an exonic region of *SLC11A2* as shown in figure (1.4 C). Both rs1048230 and rs224589 were documented to be associated with Parkinson’s disorder (Saadat et al., 2015; Wysokinski et al., 2012). Figure (1.5) shows *SLC11A2* transcripts.

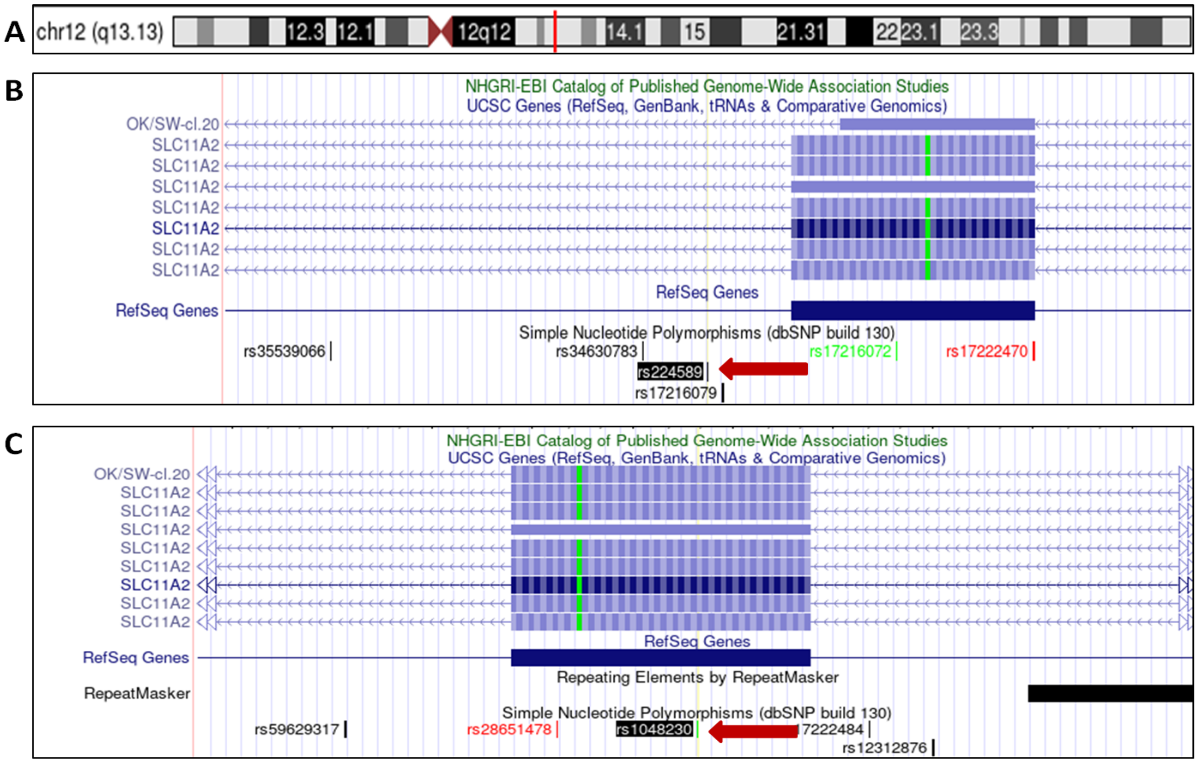

Figure 1.4. *SLC11A2* gene. A. location of *SLC11A2* gene on chr12, B. location of rs224589 SNP in *SLC11A2* gene and C. location of rs1048230 SNP in *SLC11A2* gene (Kent et al., 2002)

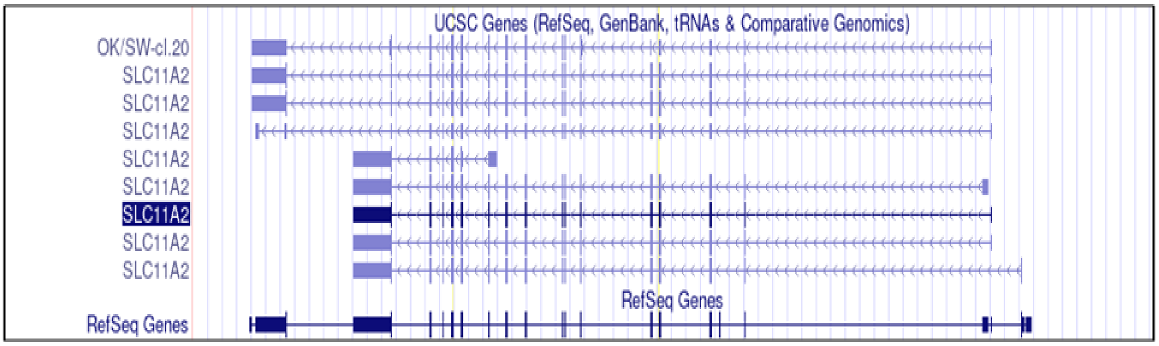

Figure 1.5. *SLC11A2* gene transcripts (Kent et al., 2002)

1.1.5.3 Solute Carrier Family 40 Member 1 (*SLC40A1*) gene.

SLC40A1 is located on chromosome 2q32.2, figure (1.6 A) (Kent et al., 2002). This gene encodes for a multipass transmembrane iron transporter, called Ferroportin-1(FPN1) that is located on the basolateral side of intestinal enterocytes and responsible for export of iron from enterocytes into the blood circulation. Ferroportin-1 is also found on the surface of hepatocytes and macrophages in which it is responsible for the release of iron into circulation (Nadadur et al., 2008). Intestinal specific inactivation of *SLC40A1* in mice resulted in iron insufficiency and accumulation of iron in enterocytes which indicate essential role of ferroportin in iron transport and homeostasis (Donovan et al., 2005). Ferroportin has been shown to be regulated at the transcription, translation and post translation levels. Several mutations in *SLC40A1* have been linked with iron overload in many studies (Mayr et al., 2010; Wallace et al., 2007; Zaahl et al., 2004). In addition to that, defects in *SLC40A1* have been associated with hemochromatosis type 4 (HFE4) (De Domenico et al., 2005). As defects in *SLC40A1* gene has been linked with iron dysregulation, this research investigated the correlation of the SNP rs1439816 which is located in an intronic region as shown in figure (1.6 B) in *SLC40A1* with ASD. Rs1439816 has been correlated with iron dysregulation in hereditary hemochromatosis (HH) (Radio et al., 2015) and has been linked with Alzheimer’s disease risk (Crespo et al., 2014). Figure (1.6 C) shows *SLC40A1* gene transcripts (Kent et al., 2002).

1.1.5.4 Hepcidin antimicrobial peptide (*HAMP*) gene.

The hepcidin antimicrobial peptide (*HAMP*) gene is located on chromosome 19q13.12, figure (1.7 A) (Kent et al., 2002) and encodes a 25 amino acid peptide. Hepcidin was found

Figure 1.6. *SLC40A1* gene. A. location of *SLC40A1* gene, B. location of rs1439816 SNP and C. *SLC40A1* transcripts (Kent et al., 2002)

to be involved in innate immunity (Ganz, 2006), in addition to that it was found that hepcidin plays a crucial role in iron homeostasis in human body (Ganz, 2006).

It has been shown that hepcidin regulates iron at both levels of absorption from the intestine and recycling by macrophages (Bayele et al., 2006; Ganz, 2006; Kemna et al., 2008; Nemeth et al., 2003). Hepcidin expression is stimulated by iron overload and inflammation, on the other hand it is suppressed by anemia and hypoxia (Nicolas et al., 2002).

Hepcidin deficiency result in iron overload in hereditary hemochromatosis, while excess hepcidin can result in iron deficiency anemia (Pandey et al., 2018). Hepcidin was found to be suppressed in patients with thalassemia syndromes (Papanikolaou et al., 2005). As defects in *HAMP* gene have been associated with iron dysregulation in many pathological conditions, in this research we investigated the correlation of the rs10421768 c.-582A>G *HAMP* promoter gene variant figure (1.7 B) with the pathogenesis of ASD. Many studies have suggested this SNP to affect iron metabolism. This SNP has been linked with iron overload in thalassemia major patients (Andreani et al., 2009). Silva has documented that this variation predisposed beta thalassemia patients to increased serum ferritin levels and iron overload (Silva et al., 2014). It was reported that this SNP may be associated with decreased hepcidin expression (Bruno et al., 2010). In vitro, the G variant decreased the *HAMP* gene transcriptional activity by 20% compared to A variant (Parajes et al., 2010). Figure (1.7 C) shows transcripts of *HAMP* gene.

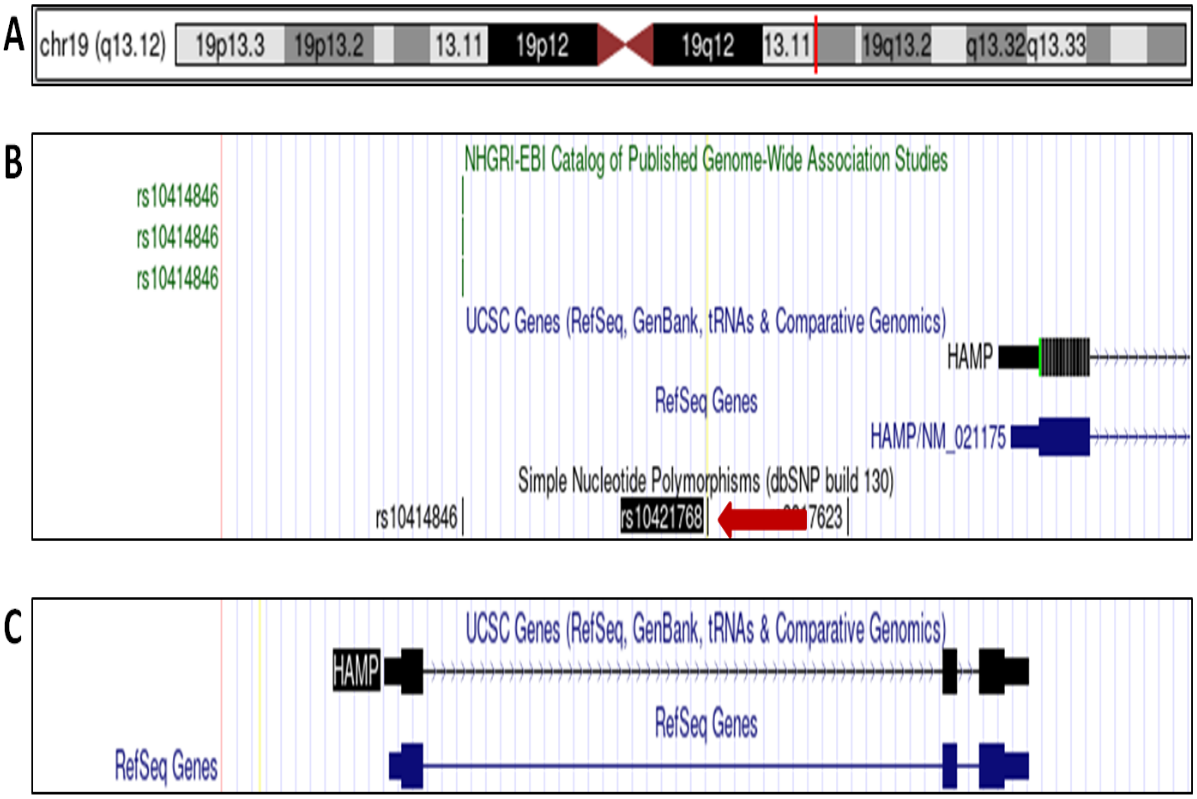

Figure 1.7. *HAMP* gene. A. location of *HAMP* gene, B. location of rs10421768 SNP and C. *HAMP* gene transcripts (Kent et al., 2002)

1.2 Statement of the problem

Iron is the most abundant transition metal in the brain, where it plays vital roles including neurotransmitter synthesis, neurons myelination, and mitochondrial function (Hare et al., 2013). Brain iron metabolism is tightly regulated and iron dysregulation is harmful for the brain both in iron overload or iron deficiency states. Autism spectrum disorder (ASD) is a group of widely spread neurodevelopmental disorder, ASD is clinically characterized with deficits in social interaction, impaired communication, and stereotyped behaviors. As these features couldn’t be diagnosed until about 3 year old, early diagnosis is a considerable challenge that encounter families and physicians. This has stressed the need for biochemical and genetic biomarkers as a tool for early diagnosis. Iron deficiency is common within autistic children, since it is estimated that nearly half of children with ASD had inadequate dietary iron intake (Herndon et al., 2009; Schmidt et al., 2014). Genetic background of iron deficiency in children with ASD is poorly investigated. On the other hand, oxidative stress has been documented to be associated with ASD and attention has been directed to investigate the etiology behind oxidative stress in ASD, since iron contributes to oxidative stress and iron-mediated oxidative stress could be significantly correlated with ASD.

1.3 Significance of the research

This thesis aimed to further study the pathophysiology and etiology of ASD among Palestinian patients, and specifically investigate genetic variations as potential early diagnostic biomarkers. The outcomes of this work may provide additional knowledge to the role of several iron gene variations in the pathophysiology of autism and introduce these variations as early diagnostic tools of the disorder.

1.4 Research Aims

This research aimed to investigate the possible correlation of specific genetic variations in major iron homeostasis genes with ASD pathogenesis. The investigated single nucleotide polymorphisms included rs11915082 which is a promoter SNP located in *TFRC* gene and was reported to be correlated with age-related macular degeneration. Two SNPs located in *SLC11A2* gene were also included, rs224589 which is an intronic SNP, and the exonic SNP rs1048230. Rs11439816 polymorphism which is an intonic variation located in *SLC40A1* was also investigated. Finally, the *HAMP* promoter SNP rs10421768 was also included.

1.5 Research hypothesis

The hypothesis of this thesis is that specific variations in specific major iron metabolism related genes functioning at the levels of iron uptake, export, and regulation affect iron homeostasis in patients with Autism Spectrum disorder (ASD) which contributes to ASD pathogenesis.

2. CHAPTER TWO: METHODOLOGY

2.1 Study population

A total of 48 Palestinian patients with ASD were enrolled in this study with a median age of 8 years. Patients were evaluated by neurologists and psychiatrists with longstanding expertise in ASD at private clinics and specialized societies. Diagnosis of probable ASD was made in accordance to guidelines of the Diagnostic and Statistical Manual of Mental Disorders by the American Psychiatric Association. A control group comprised 88 healthy individuals with no indicators of any mental disturbance or other related health problems. Both patients and control subjects were recruited from the same geographical area covering various districts in the west bank. Guardians of all patients were familiarized with the objectives and course of the research project and gave written informed consent for their children to participate in the project. Demographic and clinical characteristics of 39 patients were obtained from patients records (table 2.1) and (table 2.2).

2.2 SNP selection and genotyping

Four iron metabolism related genes were analyzed for association with ASD, with a total of 5 SNPs selected according to NCBI SNPs database as shown in (Table 2.4). The SNPs were genotyped using the RFLP-PCR for the *HAMP* gene SNP, or DNA sequencing (ABI 3500 Genetic Analyzer) for the other SNPs at the molecular genetics laboratory of the Arab American University.

2.2.1 SNPs selection

The studied SNPs were selected based on literature resources that documented the association of these SNPs with iron dysregulation in several disorders.

2.2.2 DNA isolation, qualification, and quantification

Venous whole blood samples (3-5 ml) were collected in EDTA-containing tubes, and transferred on ice to the molecular genetics laboratory of the Arab American University. Genomic DNA was isolated using the MasterPure™ Complete DNA purification Kit (Epicenter, USA) according to the manufacturer protocol as described below:

1). Five ml of whole blood were drawn into 5 ml K EDTA-containing tube and centrifuged at room temperature for 15 minutes at 1500 X g. The buffy coat (white blood cells) was withdrawn in 150 µl volume and transferred into 1.5 ml Eppendorf tubes .

2). 0.6 ml of Lysis buffer (1) (10 mM Tris-HCl, 400 mM NACL and 2 mM NA2EDTA, pH 8.2) was added to the buffy coat, mixed gently by inverting the tube 6-8 times, the bottom of the tube was flicked to suspend any remaining material .

3). Tubes were incubated for 5 minutes at room temperature, the tube was inverted 6-8 time for good mixing and then incubated for an additional 5 minutes at room temperature after flicking the bottom. The tubes were inverted 6-8 times.

4). Tubes were centrifuged for 25 seconds at 10,000 x g.

5). After centrifugation, the supernatant (lysed RBCs) was discarded leaving about 25 ul with the pellet, the pellet was vortexed for resuspension of the white blood cells.

Table 2.1 Demographic and clinical Patients data

Patient number	Society	Code	Age	Gender	Sibling	Consanguinity	Self behavior	Language skills	Self-expression
P1	Farah	M.A	6.9	Male	No	No	Normal	Yes	Normal
P2	Farah	M.D	8	Male	No	No	Aggressive	Yes	Moderate
P3	Farah	KH.H	9.4	Male	No	Yes	Normal	No	Normal
P4	Farah	A.K	7.2	Male	No	No	Normal	No	Moderate
P5	Farah	M.H	8.9	Male	No	No	Normal	No	Normal
P6	Farah	A.KH	8.8	Male	No	Yes	Normal	No	Moderate
P7	Farah	A.S	5.4	Male	No	No	Normal	No	Normal
P8	Farah	S.A	7.3	Male	No	No	Normal	Yes	Normal
P9	Farah	Y.S	4.8	Male	No	Yes	Normal	Yes	Normal
P10	Yasmine	M	9	Male	No	Yes	Aggressive	No	None
P11	Yasmine	R	5	Male	No	No	Normal	No	Minor
P12	Yasmine	M.GH	6	Male	No	No	Normal	Yes	Moderate
P13	Yasmine	S	9	Male	No	No	Normal	Yes	Normal
P14	Yasmine	A	5	Male	No	No	Aggressive	No	None
P15	Yasmine	AS	7	Male	No	No	Normal	Yes	Normal
P16	Yasmine	H	11	Male	No	No	Moderate	Yes	Normal
P17	Jod	A.A	6	Male	No	No	Normal	Yes	Minor
P18	Jod	A.M	7	Male	No	No	Moderate	Yes	Minor
P19	Jod	M	4	Male	No	Yes	Aggressive	Yes	Minor
P20	Jod	M2	8	Male	No	Yes	Moderate	Yes	None
P21	Jod	Y	4	Male	No	Yes	Moderate	No	Minor
P22	Ghad	S.R	12	Male	No	No	Normal	Yes	None
P23	Ghad	M.R	8	Male	No	Yes	Normal	No	None
P24	Ghad	A.Z	12	Male	No	Yes	Normal	Yes	None
P25	Ghad	M	11	Male	No	No	Normal	No	None
P26	Ghad	M.A 2	14	Male	No	No	Moderate	No	Normal
P27	Ghad	A.B	9	Male	No	No	Normal	No	None
P28	Ghad	W	9	Male	No	No	Normal	No	None
P29	Ghad	A.B	10	Male	No	No	Normal	Yes	Minor
P30	Ghad	Y	8	Male	No	No	Normal	Yes	Normal
P31	Ghad	Y.Z	16	Male	Yes	No	Moderate	Yes	Normal
P32	Ghad	A11	N/A	Male	N/A	N/A	N/A	N/A	N/A
P34	Rawan	B.S	6	Male	No	No	Moderate	No	None
P35	Rawan	B	9	Male	No	No	Moderate	Yes	Normal
P36	Rawan	N.A	5	Male	No	No	Normal	No	None
P37	Rawan	A7	N/A	Male	No	No	Normal	No	None
P38	Rawan	A8	N/A	Male	N/A	N/A	N/A	N/A	N/A
P39	Amal	H.D	12	Male	N/A	N/A	N/A	N/A	N/A
P40	Amal	A3	12	Male	No	No	Normal	No	None
P41	Amal	M3	9	Male	No	No	Normal	No	None
P42	Amal	A5	9	Male	No	Yes	Moderate	No	Normal

Table 2.1 Continued

P43	Amal	A6	8	Male	No	No	Aggressive	Yes	None
P45	Amal	A10	N/A	Male	N/A	N/A	N/A	N/A	N/A
P46	N/A	N/A	N/A	Male	N/A	N/A	N/A	N/A	N/A
P47	N/A	N/A	N/A	Male	N/A	N/A	N/A	N/A	N/A
P48	N/A	N/A	N/A	Male	N/A	N/A	N/A	N/A	N/A
P49	N/A	N/A	N/A	Male	N/A	N/A	N/A	N/A	N/A
P50	N/A	N/A	N/A	Male	N/A	N/A	N/A	N/A	N/A

Table 2.2. Summary of patients data

Patients	48 (clinical data available for 39)
Median age	8 years
Siblings (n)	39
Yes	1
No	38
Consanguinity (n)	39
Yes	10
No	29
Self-behavior (n)	39
Normal	25
Moderate	8
Aggressive	6
Language skills (n)	39
Yes	20
No	19
Self-expression (n)	39
Normal	14
Minor	6
Moderate	4
None	15

6). 300 µl of Lysis buffer (2) (10% SDS, protease K solution,1mg protease K in 1% SDS and mM Na2EDTA] were added to the tubes, then the cells were pipetted up and down 5-7 times for complete suspension and lysis.

7). 250 ul of Precipitation Solution was added to the previous mixture, mixed vigorously by vortex, then centrifuged for 10 minutes at 10,000 x g.

8). The supernatants were transferred into new clean microfuge tubes.

9). 700 ul of ice-cold isopropanol was added to precipitate the DNA after gentle mixing.

10). Tubes were centrifuged at 4° C for 10 minutes at 10,000 x g for DNA to be pelleted.

11). Supernatants were carefully discarded without dislodging the pellets, washed by absolute ethanol, and dried in the air.

12). DNA was resuspended in 100 µl sterile distilled water.

13). Purified suspended DNA was stored at -20° C until further use for genomic analysis.

DNA was tested for quality by gel electrophoresis using 1% agarose gel, prepared by dissolving 1 gm agarose in 100 ml Tris-Acetate-EDTA (TAE) buffer. DNA purity and concentration were tested using the nanodrop (Nanodrop 2000 C), all samples had an A260/A280 ratio above 1.7.

2.2.3 Genotype determination

The genotyping was performed using DNA sequencing or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. Polymerase chain reaction (PCR) was carried out to amplify the targeted area of the gene, each reaction tube contained 12.5 µl ready mix (lyophilized mixture of Taq polymerase, Mgcl2, dNTP and buffer), 9.5 µl distilled water, 1 µl template genomic DNA (about 200 ng/µL), 1 µl Reverse primer (final concentration of 10µM), and 1 µl Forward primer resulting (final concentration of 10µM) in 25 µl reaction volume. The sequences of primers are given in (table 2.3). PCR was run on FlexCycler2 thermocycler (Analytik Jena, Germany). After thermal cycling, PCR product was run on a 2.5% agarose gel, electrophoresis was run at 100 V for 40 minutes in TAE buffer. Thermal cycling conditions for each SNP are given in (Table 2.4).

2.2.3.1 PCR-RFLP

PCR-RFLP was used to investigate the *HAMP* gene. After amplification using PCR, fragments containing the Rs10421768 polymorphic site were digested for 24 h with HpyCH41V allele-specific restriction enzyme. Each reaction contained, 10 µl PCR product, 1.5 µl 10 x enzyme buffer, 0.2 µl (54 units of enzyme), and 3.3 µl distilled water. After incubation at 37 °C for 24 h, DNA fragments were separated on a 2.5% agarose gel, electrophoresis was run at 100 V for 40 minutes in TAE buffer. After separation, gels were analyzed by the ChemiDoc imaging system (BIO-RAD, USA). Allele and genotypes were interpreted based on specific digestion pattern.

2.2.3.2 DNA sequencing analysis

For *TFRC*, *SLC11A2*, and *SLC40A1* variants investigation, Sanger's sequencing was used. After DNA amplification, PCR product was cleaned up by mixing 5 µl of PCR product with 1 µl of clean up reagent Eppic Fast (A&A biotechnology) and incubated at 37°C for 15 minutes. Samples that required dilution were diluted using distilled water.

Table 2.3. SNPs details, sequences of primers and method of investigation

Gene	SNP reference	Position (GRCh38.p12)	Gene region	Variation	Forward primer	Reverse primer	Product size	Method used
TFRC	Rs11915082	chr3:196082268	Promoter	NC_000003.12:g.196082268G>A	GTCACTTCCTGAGGCACGTA	CGCAGTGCAATATCCAACAT	306	DNA sequencing
SLC11A2	Rs1048230	chr12:50992283	Exonic	NC_000012.12:g.50992283A>G	TCCCATTCTTCTGAGGTCTCTC	AGACCACAACCATGCCTCTG	366	DNA sequencing
SLC11A2	Rs224589	chr12:51005267	Intronic	NC_000012.12:g.51005267T>A NC_000012.12:g.51005267T>C NC_000012.12:g.51005267T>G	TGTGAGGCTGGATTTTGTTG	AGATTTTGCACATGACCTGCT	483	DNA sequencing
SLC40A1	Rs1439816	chr2:189579904	Intronic	NC_000002.12:g.189579904C>A NC_000002.12:g.189579904C>G	TGGGGAAAGATCTTCGATG	GTACGTGGTTTGTCCTGCAA	367	DNA sequencing
HAMP	Rs10421768	chr19:35281996	Intron Variant/5' UTR variant	NC_000019.10:g.35281996A>G	CCCAGGCTAGTCTTGAACTTCTG	AGGGAACACTAGATAGCCCTGAG	244	RFLP-PCR

Sanger’s sequencing was performed using the BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher scientific, USA) and run on the Applied Biosystems 3500 Genetic Analyzer. Nucleotide variations were examined using the Sequence Scanner Software 2.

Table 2.4. PCR Protocols

SNP	Initial denaturation (5 min)	Cycling			Final extension (5 min)
		Denaturation 30 sec	Annealing 30 sec	Elongation 45 sec	
Rs11915082	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
Rs1048230	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
Rs224589	94 ◦C	94 ◦C	56 ◦C	74 ◦C	74 ◦C
Rs1439816	94 ◦C	94 ◦C	58 ◦C	74 ◦C	74 ◦C
Rs10421768	94 ◦C	94 ◦C	58 ◦C	74 ◦C	74 ◦C

2.3 Statistical analysis.

The Hardy–Weinberg equilibrium (HWE) was evaluated to determine the representativeness of the study population. This analysis carried out using the SNPStats online tool (Solé et al., 2006).Overall differences in allele and genotype frequencies between ASD patients and non-ASD individuals were evaluated by Chi-Square ($\chi 2$) test or Fisher’s exact test using both the SHEsis online tool and SPSS software (IPM SPSS, 2017; Shi & He, 2005). Allelic associations with ASD risk were expressed as p value, odds ratio (OR), and 95% confidence interval (95% CI). A p value less than 0.05 was considered statistically significant.

2.4 Bioinformatics analysis

The functional consequences of SNPs that showed significant association with ASD were investigated by different in silico tools when appropriate, these include RegulomeDB, which is a database dedicated for noncoding SNP and annotates SNPs with known and putative regulatory elements in non-coding regions of human genome (Boyle et al., 2012). This database annotations are based on data from ENCODE project and other resources, combined together by a scoring system ranging from 1-6. A higher rank corresponds to a less functional significance (Boyle et al., 2012). In addition to RegulomeDB, the online tool HaploReg V4.1 was also used to further explore the potential role of non-coding genetic variants included in this study (Ward & Kellis, 2016).

3. CHAPTER THREE: RESULTS

The aim of this study is to investigate the possible association between selected genetic variants (single nucleotide polymorphisms SNPs) in major selected genes involved in iron metabolism including *TFRC*, which is involved in iron transport, *SLC11A2* which is involved in iron influx, *SLC40A1* which is involved in iron efflux, and the *HAMP* gene which is a key regulator of iron homeostasis with ASD. Five selected single nucleotide polymorphisms were investigated including rs11915082 (*TFRC*), rs224589 (*SLC11A2*), rs1048230 (*SLC11A2*), rs1439816 (*SLC40A1*), and rs10421768 (*HAMP*).

To determine whether the abovementioned variants were associated with ASD pathogenesis, the allelic frequencies and genotype frequencies were compared between Palestinian ASD patients (n=48), and healthy control subjects (n=88). All investigated SNPs were in accordance with Hardy-Weinberg equilibrium (HWE) in control group ($p>0.05$) at the genotype levels.

3.1 Genotyping and statistical analysis of rs11915082 (*TFRC*).

Amplification of the DNA sequence with the SNP variant rs11915082 using PCR yielded a 306 bp product as shown in figure (3.1 A) which demonstrates a representative image of the yielded single bands amplicon. Sanger’s sequencing was used for rs11915082 genotyping, as depicted in Figure (3.1 B) which shows representative figures of sequencing results.

Regarding statistical analysis, a significant difference was evident in allele frequencies between the control and case groups for rs11915082 in the *TFRC* gene (p=0.008), where the G allele was associated with increased ASD risk (OR=2.06, 95% CI= 1.19-3.55, p=0.008). At the genotype level the rs11915082 showed significant difference between cases and control (p=0.041), the rs11915082 was found to be more frequent in the homozygous GG form in the patients group compared to the control group with respective frequencies 56.2% vs. 35.2% (p=0.041), and was in agreement with allele association analysis in which the G allele was associated with increased risk of ASD development.

3.2 Genotyping and statistical analysis of rs224589 (*SLC11A2*).

An amplicon of 483 bp was yielded by the amplification using PCR which included the indicated SNP variant as shown in b (3.2 A). Figure (3.2 B) shows representative figures of the sequencing results. Statistical analysis showed no significant difference of both allele and genotype frequencies between cases and controls p=0.584 (table3.1) and p=0.194 (table3.2) respectively.

3.3 Genotyping and statistical analysis of rs1048230 (*SLC11A2*).

As shown in figure (3.3 A), the PCR product showed clear single bands with a size of 366 bp as expected. Sanger’s sequencing was used for the variant rs10482302 genotyping. Figure (3.3 B) shows representative sequencing results. Statistical analysis showed no significant difference in both allele and genotype frequencies between cases and controls p=0.635 and p=0.223 respectively.

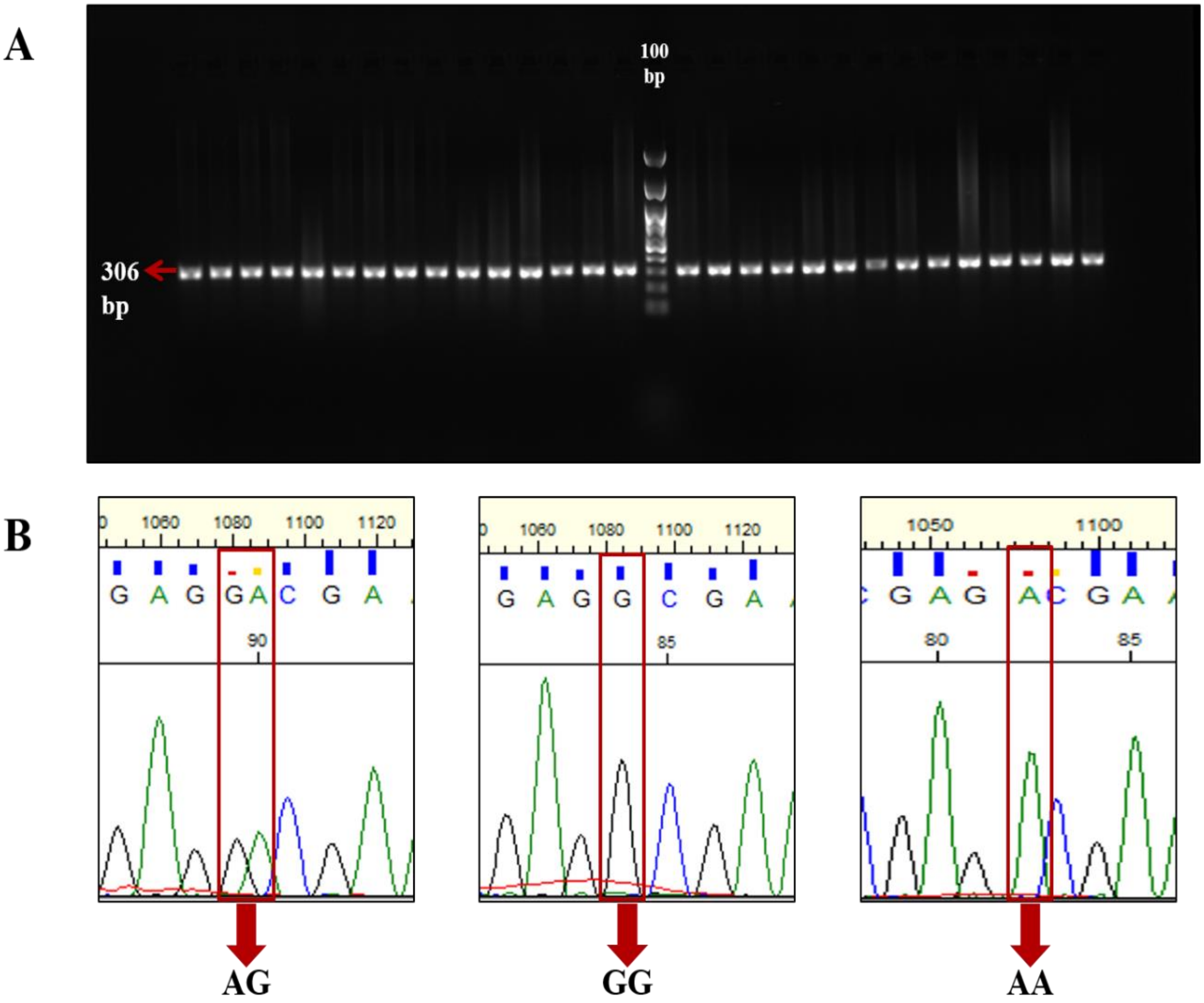

Figure 3.1. Genotyping of the rs11915082 variant. A. Agarose gel showing the indicated amplified DNA fragments (306 bp) containing the rs11915082 variant and B. Representative genotype sequence showing the obtained sequence with AG, GG, and AA genotype.

3.4 Genotyping and statistical analysis for the rs1439816 SNP variant (*SLC40A1*).

Amplification of the rs1439816 containing sequence using PCR yielded a 367 bp product as shown in figure (3.4 A) which shows a representative image of the yielded amplicon as qualified using gel electrophoresis, showing clear bands. Sanger's sequencing was used for rs1439816 genotyping, figure (3.4 B) shows representative figures of sequencing results.

A significant difference was seen in allele frequency between controls and cases for Rs1439816 (*SLC40A1* gene) ($p < 0.0001$). The C allele of Rs1439816 obviously showed strong significant association with increased disease risk (OR= 5.13, 95% CI=3.00-8.79, $p < 0.0001$).

At the genotype level , a significant difference was seen between controls and cases $p=0.000$ (table3.2) with the CC genotype being the most frequent in patients compared to controls with respective frequencies 47.9% vs. 8% (table3.2), and this was also in agreement with allele association analysis, as C allele showed to be associated with ASD increased risk.

3.5 Genotyping and statistical analysis of rs10421768 (*HAMP*)

PCR amplification of rs10421768 containing sequence resulted in a product with 244 bp size as shown in figure (3.5 A). Genotyping of Rs10421768 variant was carried out by RFLP-PCR which yielded two DNA fragments (187 and 57 bp) for the A allele, and a single DNA fragment (244bp) for the C allele following digestion with the (HpyCH41V) enzyme, figure (3.5 B).

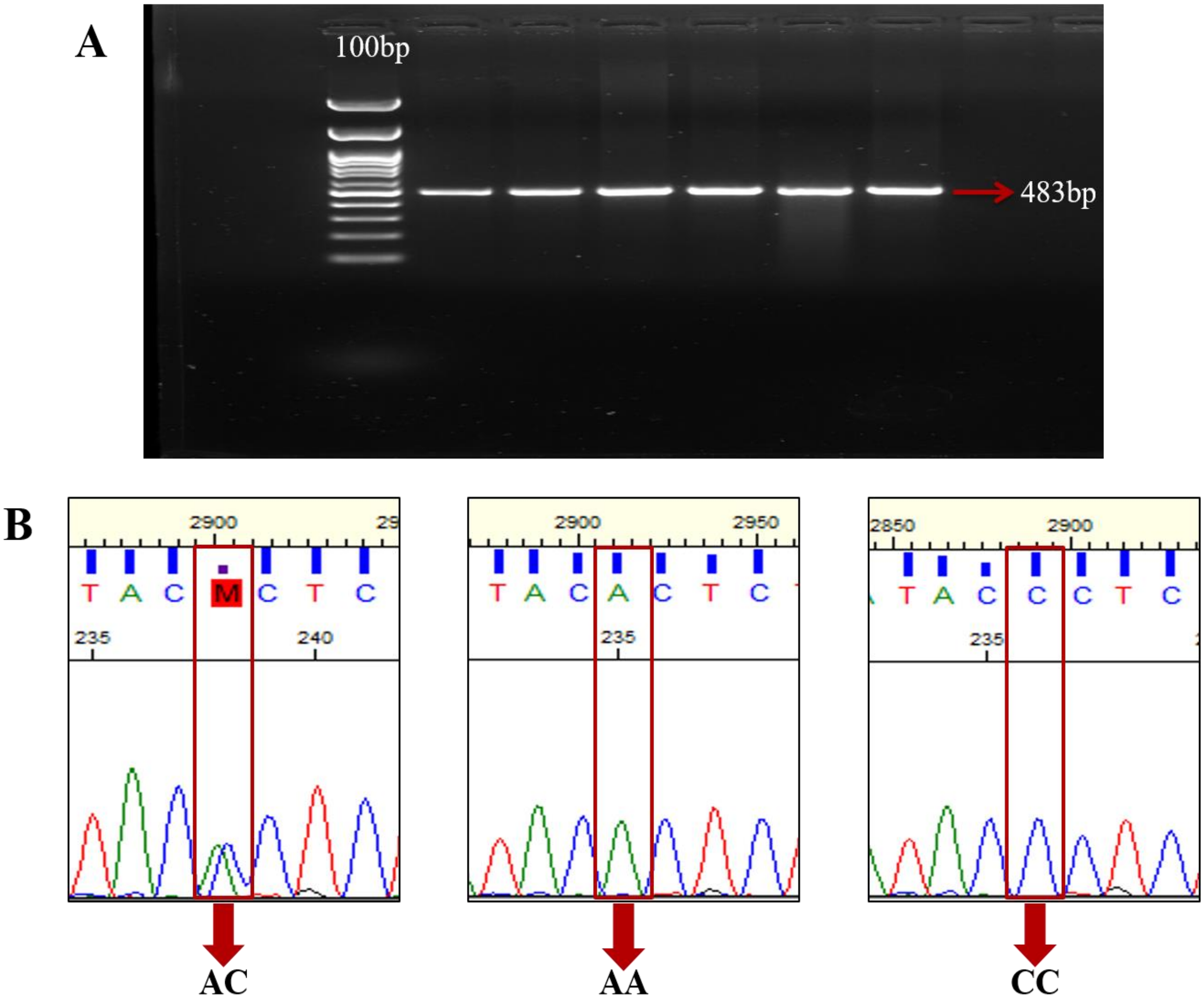

Figure 3.2. Genotyping of the rs224589 variant. A. Agarose gel showing the indicated amplified DNA fragments (483 bp) containing rs224589 variant and B. Representative genotype sequence showing the obtained sequence with AC, AA and CC genotype.

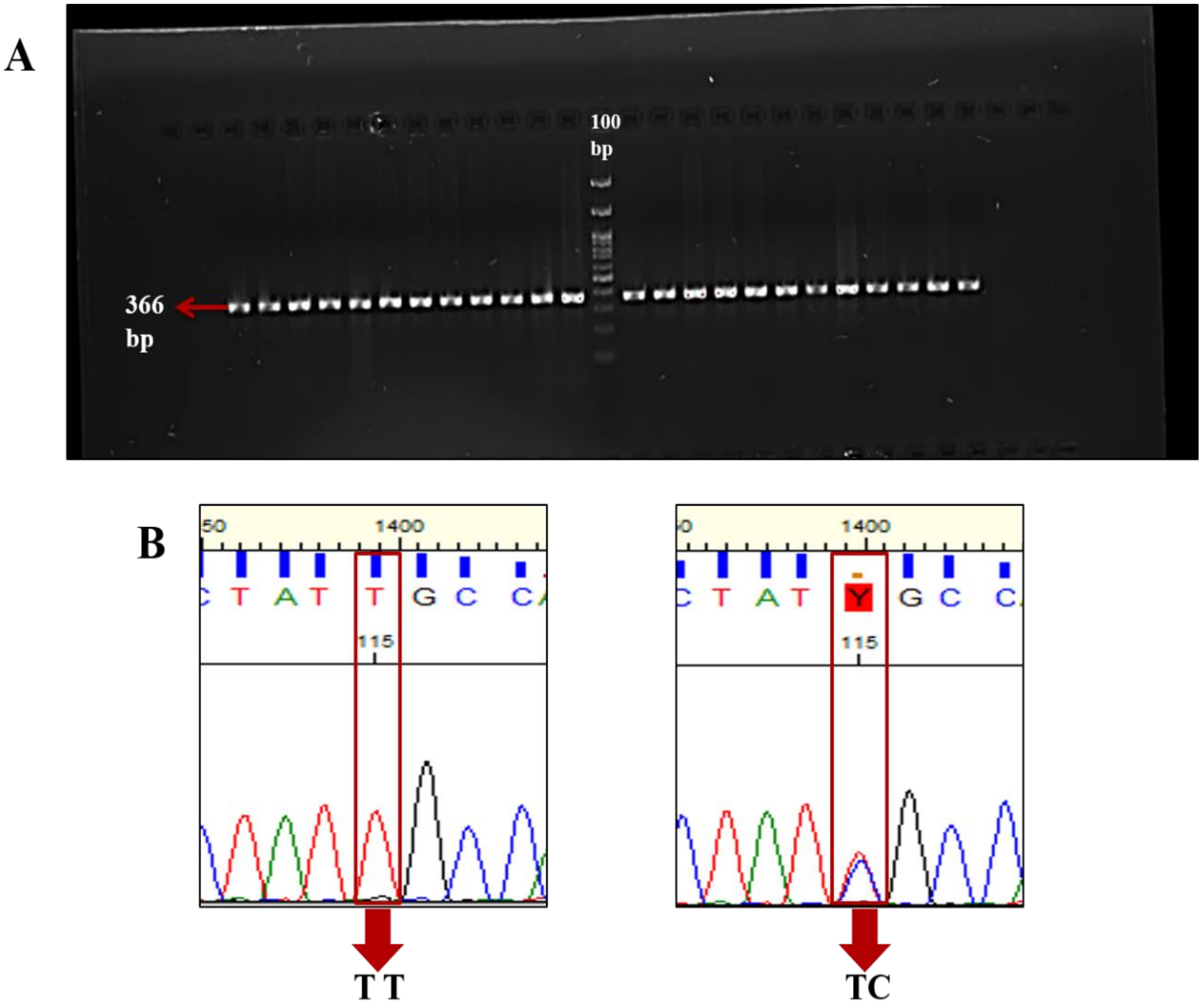

Figure 3.3. Genotyping of the rs1048230 variant. A. Agarose gel showing the indicated amplified DNA fragments (366 bp) containing the rs1048230 variant and B. Representative genotype sequence showing the obtained sequence with TT and TC genotype.

Sanger's sequencing was performed to confirm the genotypes of Rs10421768 SNP for randomly selected samples as demonstrated in Figure (3.5 C).

Statistical analysis showed no significant difference in allele frequencies between cases and controls (p=0.393). On the other hand, a significant difference in genotype frequencies was seen between cases and controls (p=0.028), with the GG genotype being in high frequency in patients in comparison to controls 10.4% vs. 1.1%.

3.6 Association of allele and genotype frequencies of SNPs with the overall phenotype of ASD.

In summary, tables 3.1 and 3.2 demonstrate the allele frequencies and genotype frequencies of all investigated variants. Significant differences were seen in alleles frequencies between the control and case groups for SNPs rs11915082 (*TFRC* gene) (p=0.008) and rs1439816 (*SLC40A1* gene) (p<0.0001) (table 3.1). Regarding Rs11915082 the G allele showed to be associated with increased ASD risk (OR=2.06, 95% CI= 1.19-3.55, p=0.008) (table3.1).On the other hand, the C allele of Rs1439816 showed to be associated with increased disease risk (OR= 5.13, 95% CI=3.00-8.79, p<0.0001) (table3.1). Both rs224589 and rs1048230 (*SLC11A2*) showed no significant differences in allele frequencies between cases and controls.

At the genotype level, our results indicated that the polymorphisms with significant association with the increased risk of ASD were rs11915082 (p=0.041), rs1439816 (p=0.000), and rs1042176 8 (p=0.028) (table3.2). The rs11915082 variant was found to be more frequent in the homozygous GG form in the patients group compared to the controls with respective frequencies 56.2% vs. 35.2% (p=0.041), and was in agreement with allele

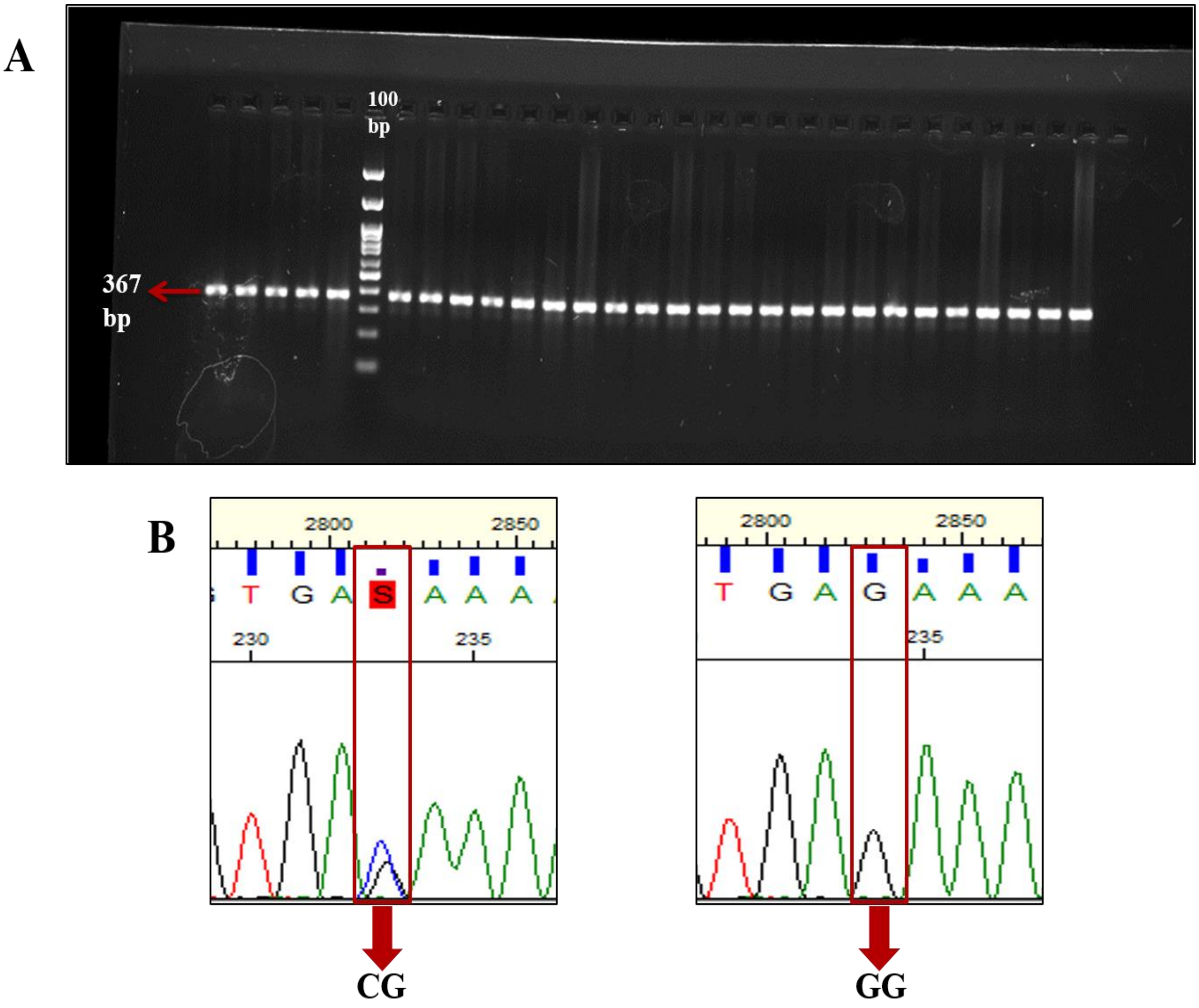

Figure 3.4. Genotyping of the rs1439816 variant. A. Agarose gel showing the indicated amplified DNA fragments (367 bp) containing the rs1439816 and B. Representative genotype sequence showing the obtained sequence with CG and GG genotype.

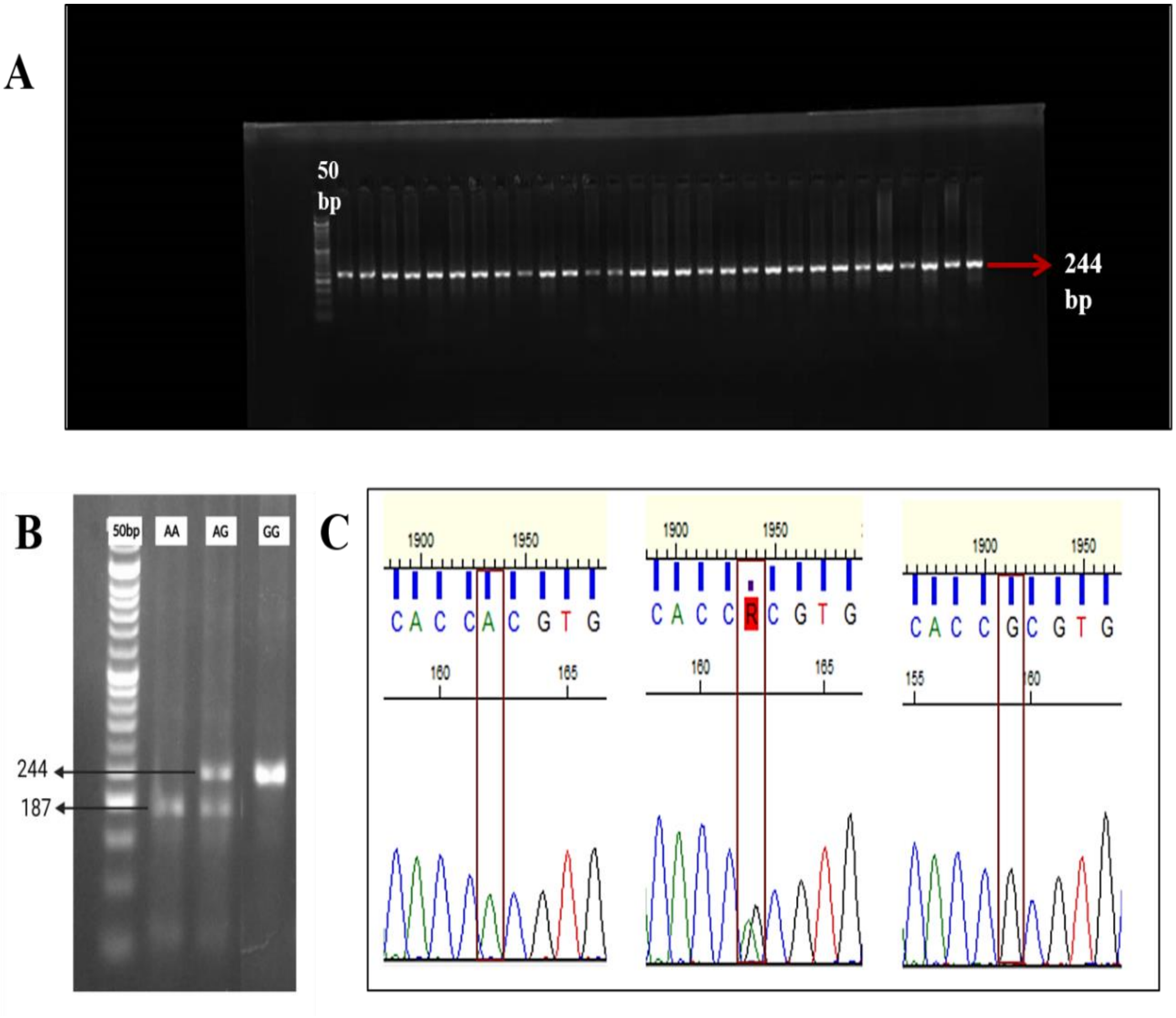

Figure 3.5. Genotyping of the rs10421768 variant. A. Agarose gel showing the indicated amplified DNA fragments (244 bp) containing the rs10421768 genotype. B. Representative image of RFLP results with the AA genotype resulted in two bands (187 bp and 57 bp), the AG genotype resulted in two bands (244 and 187) and the GG genotype resulted in one band (244 bp). C. Representative genotype sequence showing the obtained sequence with AA, AG, and GG genotype.

association analysis in which the G allele showed a significant association with ASD increased risk. Regarding the variant rs1439816, the CC genotype was more frequent in patients compared to controls with respective frequencies 47.9% vs. 8%, and this was in agreement with allele association analysis, as C allele showed to be associated with ASD increased risk. On the other hand, the polymorphic site rs10421768 showed no significant difference at the level of allele, but a significant difference at the genotype level, with the GG genotype being in high frequency in patients in comparison to controls 10.4% vs. 1.1% (table3.2). In addition to association analysis, SNP-SNP interaction analysis using logistic regression showed that rs1439816 (FPN) had the most significant effect.

Association analysis was carried out to investigate whether there is possible correlation between each SNP and three core patients' characteristics. Those characteristics included self-expression, language skills, and self-behavior. Results indicated that rs224589 (DMT1) was associated with self-expression characteristic ($p=0.026$), and rs1439816 (FPN) was associated with self-behavior characteristic ($p=0.003$).

Table 3.1 Statistical analysis of allele frequencies

SNP	Allele	Patient (%) (n=48)	Control (%) (n=88)	P Value	OR (95% CI)
Rs11915082	G	71 (74.0%)	102 (58.0%)	**0.008**	2.06 (1.19-3.55) 0.48 (0.28-0.83)
	A	25 (26.0%)	74 (42.0%)		
Rs1048230	T	74 (77.1%)	140 (79.5%)	0.635	0.86 (0.47-1.57) 1.15 (0.63-2.10)
	C	22 (22.9%)	36 (20.5%)		
Rs224589	A	32 (33.3%)	53 (30.1%)	0.584	1.16 (0.68-1.97) 0.86 (0.50-1.46)
	C	64 (66.7%)	123 (69.9%)		
Rs1439816	C	65 (67.7%)	51 (29%)	**<0.0001**	5.13 (3.00-8.79) 0.19 (0.11-0.33)
	G	31 (32.3%)	125 (71.0%)		
Rs10421768	A	75 (78.1%)	145 (82.4%)	0.393	0.76 (0.41-1.41) 1.30 (0.70-2.43)
	G	21 (21.9%)	31 (17.6%)		

Values in bold indicate significant difference

Table 3.2 Statistical analysis of genotype frequencies

SNP	Genotype	Patient (%) (n=48)	Control (%) (n=88)	P Value
Rs11915082	GG	27 (56.2%)	31 (35.2%)	**0.041**
	AG	17 (35.4%)	40 (45.5%)	
	AA	4 (8.3 %)	17 (19.3%)	
Rs1048230	TT	29 (60.4%)	53 (60.2%)	0.223
	TC	16 (33.3%)	34 (38.6%)	
	CC	3 (6.2%)	1 (1.1%)	
Rs224589	AA	6 (12.5%)	4 (4.5%)	0.194
	AC	20 (41.7%)	45 (51.1%)	
	CC	22 (45.8%)	39 (44.3%)	
Rs1439816	CC	23 (47.9%)	7 (8%)	**0.000**
	CG	19 (39.6%)	37 (42%)	
	GG	6 (12.5%)	44 (50.0%)	
Rs10421768	AA	32 (66.7%)	58 (65.9%)	**0.028**
	AG	11 (22.9%)	29 (33.0%)	
	GG	5 (10.4%)	1 (1.1%)	

Values in bold indicate significant difference

4. CHAPTER FOUR: DISCUSSION

1.4 Discussion and Conclusion

Iron is found in high concentrations within the brain, as it has a number of essential roles in different cellular processes including neurotransmitter synthesis, myelination of neurons and mitochondrial function. Brain iron is highly regulated by different mechanisms which include different genes products (Nadadur et al., 2008). Oxidative damage is being considered one of the contributing factors in ASD pathogenesis (Manivasagam et al., 2020). Brain oxidative damage could be enhanced by excess of free iron within the brain. Increased levels of non-transferrin bound iron (NTBI) may occur due to deregulation in the expression of iron homeostasis proteins including, transferrin (Tf), Transferrin Receptor (TfR1), hepcidin antimicrobial peptide gene (*HAMP*), Ferroportin (FPN) and divalent metal-ion transporter1 (DMT1). On the other hand, decreased levels of transferrin and ceruloplasmin have been documented in blood samples from autistic patients (Chauhan et al., 2004; Meguid et al., 2011; Yorbik et al., 2002; Zoroglu et al., 2004).

The possibility that ASD risk can be altered due to an individual genetic variations that affect and modify their susceptibility cannot be excluded (Aschner & Ceccatelli, 2010). Even in the absence of toxic iron levels, iron could contribute to autism etiology due to genetic susceptibility. In the present study, the association of specific single nucleotide polymorphisms in specific genes involved in iron metabolism including (rs11915082 in *TFRC* gene, rs224589 in *SLC11A2* gene (DMT1), rs1048230 in *SLC11A2* gene (DMT1), rs1439816 in *SLC40A1* (FPN), and rs10421768 in *HAMP* gene (Hepcidin).

The transferrin receptor (TfR1) together with transferrin (Tf) mediates the delivery of Tf-bound iron into cells. The rs11915082 (-253G > A) polymorphism is located in the 5’-upstream region (promoter) of the *TFRC* gene, it is expected to affect the regulation and level of expression of this gene. This locus is documented to be linked to the erythrocyte phenotype(mean hemoglobin volume MHV) (Ganesh et al., 2009). This SNP was documented to be associated with age-related macular degeneration (Wysokinski et al., 2015). In silico analysis of this SNP using the RegulomeDB database showed that this SNP has a score of 4, meaning that there is a lack of evidence that this variation actually has a regulatory consequence (Boyle et al., 2012). Alternatively, HaploReg analysis showed that this variation affect the binding sites of the regulatory factors CHD2, SRF, and ZBTB33 (Ward & Kellis, 2016). Our study demonstrate that the rs11915082 genetic variant is correlated with ASD pathogenesis, with the GG genotype being more frequent in patient group compared to control group. Our results indicate the role of this SNP in ASD pathogenesis, we predict the effect of this SNP to be through the disruption of iron metabolism in the brain. Even though the G allele is the most frequent allele in all ethnic groups, it showed to be associated with ASD risk. That can be explained by the fact that ASD is a complex polygenic disorder, hundreds of genes may be associated with ASD risk, and allele effect vary between individual depending on the genetic variations on other genes.

Ferroportin (FPN) mediates the export of iron from cells and is encoded by the *SLC40A1* gene. The rs1439816 SNP is located in intron 1 of the *SLC40A1* gene. A study on this variant reported the C allele as a risk factor of Alzheimer’s disease, and was significantly associated with decreased expression of the *SLC40A1* gene in those patients, causing increased Fe levels inside the cell (Crespo et al., 2014). Our results similarly showed that the C allele was significantly more frequent in ASD cases compared to controls. In silico analysis using RegulomeDB showed that this SNP has a score of 4, meaning that there is a lack of evidence that this variation actually has a regulatory consequence. However, HaploReg analysis showed that this variation disrupt two regulatory motifs including the binding sites of Eomes and SREBP (Ward & Kellis, 2016). Our study showed a strong significant difference in the ferroportin genetic variant rs1439816 frequencies between controls and patients which indicates a possible role of this SNP in ASD pathogenesis.

The divalent metal transporter 1 (DMT1) is encoded by the solute carrier family 11 member 2 gene *SLC11A2* and is ubiquitously expressed in different tissues including the brain and the liver (Gunshin et al., 1997). DMT1 is involved in iron absorption and transport. The SNP rs1048230 (1254T>C) is a synonymous variant located in the coding region of *SLC11A2* gene (Kent et al., 2002) and does not result in amino acid substitution. This SNP was reported in ClinVar as a benign variant when studied with iron overload in hypochromic microcytic Anemia (Accession: VCV000309312.2) (Landrum et al., 2018). The TT genotype and T allele were documented to be associated with Parkinson's disorder (Saadat et al., 2015). Regarding the rs224589 polymorphism (IVS4+44 C > A) which is an intronic variant located in intron 4, the C allele was reported to be associated with Wilson's disease, age related macular degeneration, and Parkinson's disorder (He et al., 2011; Przybyłkowski et al., 2014; Wysokinski et al., 2012). It is speculated that the rs224589 polymorphism may affect alternative splicing or constitutive splicing of *SLC11A2* gene through the corruption of splicing regulatory cis-elements, which can result in incorrect isoforms of DMT1 (Przybyłkowski et al., 2014; Tazi et al., 2009; Ward & Cooper, 2010).

In addition, since this SNP is located in an intronic region, it might affect the transcription, post-transcription, and ultimately the mRNA translation of *SLC11A2* gene. Our results showed that the both SNPs rs1048230 and rs224589 located in the *SLC11A2* gene were not correlated with ASD pathogenesis as both alleles and genotypes frequencies were not significantly different between patient and control groups.

Hepcidin is encoded by the hepatic antimicrobial protein gene *HAMP* and is considered as the central regulator of systemic iron homeostasis through the control of FPN1 expressions (Fuqua et al., 2012). The rs10421768 polymorphism (c.-582 A>G) is located in the promoter region of the *HAMP* gene. This variation has been reported to be associated with many disorders. This SNP affects a conserved non-coding transcriptional box of the promoter of the *HAMP* gene and it may affects its binding affinity to transcriptional factors or the response to these factors. In addition, it is suggested that this variation results in the activation of the *HAMP* gene expression by both upstream regulatory factor 1 and 2 (USF1/USF2) and cMyc/Max heterodimers that occur through E box within the promoter, which can alter the regulation of the *HAMP* gene and hepcidin function in iron homeostasis (Bayele et al., 2006; Bayele & Srai, 2009). This polymorphism may constitute a risk factor for increased iron levels in patients with ASD. In silico analysis using RegulomeDB showed that this variation has a score of 2b, meaning that this variation is likely to affect transcription factor binding (Boyle et al., 2012). In addition, the HaploReg analysis showed that this SNP affects the binding sites of a group of factors such as Myc, E2A, and HIF1 (Ward & Kellis, 2016). Our results indicated the role of the *HAMP* gene SNP rs10421768 in the pathogenesis of ASD, this SNP showed significant differences in genotype frequencies between patient and control groups, but no significant difference was found in alleles frequencies between the two groups, and this can be explained by the relative small size of patients group.

This study is the first study to draw the attention toward the role of iron dysregulation in ASD pathogenesis. Effects of genetic variations in iron homeostasis genes on iron homeostasis is evident, but in light of the available published data they play minor role (Benyamin et al., 2014; Stickel et al., 2014). Our study was focused on molecular genetic analysis. It is highly recommended to expand the investigation on the relationship of iron metabolism-related genes polymorphisms and various hematological parameters such as: serum iron, ferritin levels, transferring levels and total iron blood capacity (TIBC). In addition, our data strongly indicate the need to directly investigate the iron situation in brain tissues of autistic patients. In addition, the application of individual SNPs association studies is considered limited due to the low penetrance of individual SNPs, and difficulty in their expressivity identification. Therefore the application of haplotypes association is considered more valuable in studying genetic variations association with a specific phenotype (Crawford & Nickerson, 2005).

Our in silico analysis suggested that the SNPs that gave significant correlation with ASD risk may affect the binding of many transcription factors. Considering that, we recommend this aspect to be investigated further. A possible approach for that is by looking at those factors’ binding sites, then investigate whether these sites are located within the SNPs regions. If positive results were indicated, then binding and functional analysis can be done to experimentally investigate the effects of the mentioned SNPs.

In conclusion, iron is critical for proper neurodevelopment and its metabolism in the brain is tightly regulated, thus polymorphisms within iron metabolism genes may be linked to ASD phenotype. The results generated from this study give significant insights into the association of genetic variations in major iron metabolism genes and ASD development, indicating the role of iron dysregulation in ASD development and pathogenesis. This study provides possible novel genetic markers for ASD susceptibility that can be incorporated in early diagnostic genetic analysis of ASD as our results showed a strong association of different SNPs with ASD including rs11915082 in the *TFRC* gene, rs1439816 in the *SLC40A1* gene and rs10421768 in the *HAMP* gene. Further analysis on large cohorts is needed to further investigate and strongly indicate the significance of these genetic markers.

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الملخص

يعرَف إضطراب طيف التوحد بأنه مجموعد متنوعة من الأمراض التي تؤثر على النمو العصبي و التي تعود لأسباب جينية و بيئية. في الوقت الراهن يعتمد تشخيص إضطراب طيف التوحد على المعايير السريرية مما يشكل حاجة ملحة لوجود فحوص وراثية تساعد في التشخيص المبكر للمرض. بما أن الحديد يلعب دوراً محورياً في نمو الدماغ، و توليف الناقل العصبي، و تكوين الغلاف الميليني في الخلايا العصبية، و وظائف عضيات الميتوكوندريا فقد إفترضنا أنَ حدوث خلل في تنظيم الحديد في الدماغ قد يلعب دوراً في حدوث و تطور أمراض إضطراب طيف التوحد. إن هدف هذه الدراسة يتمحور حول دراسة وجود علاقة بين بعض التغيرات المفردة في القواعد النيتروجينية في مجموعة محددة من الجينات التي تلعب دورا مهما في أيض و تنظيم الحديد و مرض إضطراب طيف التوحد. و قد شملت على المتغيرات التالية, rs11915082 (*TFRC*),rs1048230 (*SLC11A2*), rs224589 (*SLC11A2*), rs1439816 (*SLC40A1*), rs10421768 (*HAMP*). اشتملت هذه الدراسة على 48 مريض فلسطيني مصاب بإضطراب طيف التوحد و 88 شخص سليم. لتحديد التغيرات الجينية في كل موقع جيني لكل مشارك في هذه الدراسة تم استخدام تقنيتي (RFLP-PCR) و (Sanger’s sequencing).

كشفت بيانات هذه الدراسة أن التغير الجيني (G) (rs11915082) ($p=0.008$) و التغير الجيني (C) (rs1439816)) ($p<0.0001$) قد إرتبطا بوجود المرض. أما على مستوى النمط الجيني، أظهرت المواقع الجينية (rs11915082, $p=0.041$)، (rs1439816, $p=0.000$)، و (rs10421768, $p=0.028$) ارتباطاً كبيراً بوجود المرض.

تظهر مخرجات هذه الدراسة وجود دور للتغيرات الجينية في بعض الجينات التي تلعب دوراً مهماً في عملية إستقلاب الحديد في حدوث إضطراب طيف التوحد وتشير إلى أنَ بعض الإختلافات الوراثية يمكن استخدامها كمؤشرات وراثية للتشخيص المبكر.