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Identification of miRNA as a Biomarker for the Efficient Diagnosis of Autism Spectrum Disorder

by

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I dedicate this thesis to my parents and my teachers.

CERTIFICATE OF APPROVAL

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Ayesha Safdar

Abstract

Autism Spectrum Disorder (ASD) is a complex diverse group of neurodevelopmental disorders including disturbances in social skills, verbal and non-verbal communications, repetitive and restricted behavior, activity or response to which the scientific community has paid great attention in the last decade. The pathogenesis of ASD is still unknown, but several studies have reported gene-environment interactions in the onset of disease. In an endeavor to explore the basic causes of ASD for early diagnosis and treatment, efforts have been made to identify reliable biomarkers. Biomarkers may have the significant worth in early potency and safety assessments like invivo studies in case of animal models, invitro studies in tissue sampling and may be highly beneficial in early phase clinical trials to demonstrate feasibility and realization. Biomarkers can be of any type like gene, miRNA, mRNA, hormone or any biomolecule in body. Biomarkers have to be chosen very carefully because they can be specific for specific disease. MicroRNAs are small, regulatory noncoding regions of RNA that modulate expression of gene through posttranscriptional mechanisms by inhibition or degradation of specific mRNA targets. miRNAs have important role in physiological pathological processes diversity. Furthermore, miRNAs are more stable than mRNAs and also barely susceptible to slight differences in processing of samples. The above mentioned attributes make them remarkable biomarker candidates. Effectively and easily accessible sources of body fluids such as serum, blood, saliva or urine would provide an ideal source to miRNA biomarkers [1]. In this study insilico techniques are used to identify novel biomarkers. The analysis identified the efficient diagnostic biomarkers(miR-106b-5p,miR-93-5p) associated with Autism Spectrum Disorder which can aid in earlier treatment as miRNAs take part in significant roles in central nervous system development, and function.

Keywords: Autism Spectrum Disorder (ASD), Biomarker, MicroRNAs, Insilico Techniques.

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Abbreviations

ACPA	Anti-cyclic Citrullinated Protein Antibodies
ASD	Autism Spectrum Disorder
AutDB	Autism Informatics Portal
CNVs	Copy Number Variants
DAVID	Database for Annotation, Visualization and Integrated Discovery
FQS	Friendship Qualities Scale
HCG	Human Chronic Gonadotropin
HMDD	Human microRNA Disease Database
KEGG	Kyoto Encyclopedia of Genes and Genome
miRNA	MicroRNA
OMIM	Online Mendelian Inheritance in Man
QSQ	Quality of Socialization Questionnaire
RF	Rheumatoid Factor
SIAS	Social Interaction Anxiety Scale
SSRS	Social Skills Rating System
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
WHO	World Health Organization

Chapter 1

Introduction

Autism Spectrum Disorder is a complicated and heterogeneous neurodevelopmental disorder that influences the developmental paths in many key behavioral domains, along with the social interactions, psychological features and language impairment. This disorder includes problems in socialization and repetitive behavior patterns. The term "spectrum" indicates to the broad range of symptoms and intensity associated with the Autism spectrum disorder (ASD) subjects.

Autism spectrum disorder incorporates conditions that were formerly viewed as isolated such as autism, childhood disintegrative disorders, Aspergers's syndrome and an unidentified character of pervasive developmental disorder. The fundamental brain dysfunction that prompts the social qualities is not surely known. Complicated mental disorders cannot simply be delineated as being related to underconnectivity or over connectivity, however could involve some assortment of anomalous connectivity that fluctuates between totally different regions of brain [2].

The prevalence of Autism spectrum disorder has rapidly raised going up to 1:37 children in the U.S in recent past [3]. Literature reports that the ASD influences approximately 1-2% of the population and the ratio of male to female is 4-5:1 [4]. The severity of autistic characteristics along with occurrence of comorbid illnesses which incorporate anxiety, intellectual incapacity, epilepsy, and disorders related to gastrointestinal substantially vary between autistic individuals [5][6][7][8].

Table 1.1: Early Symptoms of Autism Spectrum Disorder [9]

Early Symptoms		
·	By age of 12 months	Child does not respond to name.
·	By age of 14 months	Does not show interest by pointing objects.
·	By age of 18 months	Does not like to play.
·	General	Escape from eye contact and prefer to stay alone.
		Does not understand others feelings and also not able to describe own.
		Delayed speech and communication skills.
		Repetitive behavior e.g. repeats phrases or words again and again.
		Gives unrelated answer to any question.
		Gets upset by minor changes.
		Child has obsessive interests.
		Shows repetitive movements e.g. flapping hands, spinning in circles.

ASD is formerly identified by distinguishing usual behaviors in autistic individual [9]. Though skilled clinicians can diagnose ASD in children with age 24 months, the typical age at which the disorder is diagnosed remains significantly high and reaches to 04 years [3].

Families usually wait for an extended period of time preliminary to sustain a complete diagnosis because of the limited number of competent clinicians capable of executing a meticulous and pragmatic assessment [10]. The diagnostic criterion used for assessment of ASD is DSM-5.

The DSM-5 criterion has been used to accurately identify ASD in younger children and those with mild symptoms. In this criteria core symptoms are divided into two main domains such as social communication and restrictive and repetitive behaviors [11].

Table 1.2: DSM-5 Criteria for ASD [11]

Domains	**Criteria**	**Example**
A. Social interactions and Social communication persistent deficits. Must have all three symptoms in following domain.	1. Social-emotional reciprocity	· Reduced sharing of interests, emotions. Failure to respond social interactions. Poor socialization.
	2. Nonverbal communication behaviors used for socialization.	· Avoid eye contact, no facial expressions. Poorly integrated verbal and non verbal communication skills. · Difficulty in sharing own feelings and understanding others. Repeat words or phrases. Also difficulty in making friends.
	3. Repetitive motor movements, object usage, speech	
B. Repetitive or restricted behavior, interests. Must have two of the four symptoms.	1. Repetitive motor movements, objects.	Motor stereotypies, echolalia.
	2. Ritualized patterns of verbal and nonverbal behavior.	
	3. Restricted or fixated interests.	· Upset by small changes.
	4. Unusual interests in sensory aspects of environment.	· Strong attachment with unusual objects. · Show unusual reactions to sound, smell, feel.

DSM-5 criterion is solely based on psychological patterns. However, it has been reported in recent studies that clinicians and researchers have shown concerns related to the impact of DSM-5 criteria because the complex as well as strict nature of criteria is not suitable for the early diagnosis of ASD [12]. Early diagnosis is important to restrict the onset of disorder at early stages. No doubt the intensive behavioral therapies are coping up with this issue [13][14] but it has been reported that the advantage of prompt intervention is larger the sooner the intervention

is started. So far, the etiology and pathological process of ASD have not been interpreted fully and it has been reported in many studies that ASD is a multi-factorial disorder with various risk factors ranging from genetic, epigenetic and environmental factors [4][15][16][17].

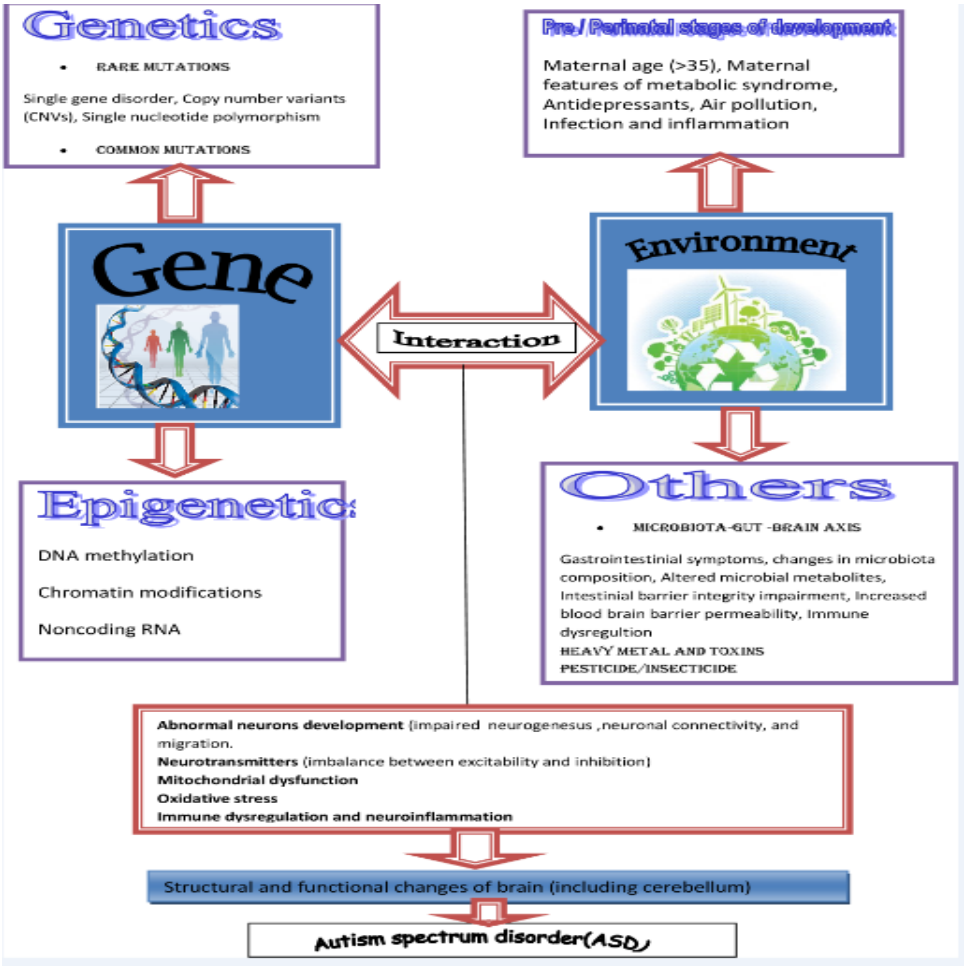

FIGURE 1.1: both the gene and environment play role in causation of ASD. Interactions of gene and environment factors give rise to certain mechanism involved in ASD, which in return cause structural and functional alterations in brain

There are no specific drugs available to treat core symptoms of ASD. So far, the attempt to search for biomedical treatment on ASD core symptoms is futile, although significant evidence suggests that early intervention can improve the outcomes of children with ASDs [18]. It has been reported that early intervention results in rapid improvement [15][16]. The early intervention strategies could only be offered if the diagnosis is made well in time as the early detection of disease is a significant step for patient care in neurodevelopmental disorders such as ASD. In

this perspective, it becomes essential to avoid delayed diagnosis but the absence of liable and specific biomarkers makes ASD very demanding in terms of early diagnosis. So, for the efficient early diagnosis of ASD a reliable and stable biomarker is required. Biomarkers can be of any type like gene, miRNA, mRNA, hormone, protein or any bimolecule in body. Biomarkers have to be chosen very carefully because they can be specific for specific disease.

Depend on the interpretation and understanding of the ASD etiological mechanisms, and according to previous studies the use of selected combinations of biomarkers have been found to be constructive for distinguishing autistic individuals from healthy ones. A biomarker has been characterized as "a feature that is objectively measured and evaluated as a pointer of normal biological processes, pharmacological responses or pathogenic processes to a therapeutic intervention"[19].

Biomarkers may have the significant worth in early potency and safety assessments like invivo studies in case of animal models, invitro studies in tissue sampling and highly beneficial in early phase clinical trials to demonstrate feasibility and realization. Biomarkers have many other productive applications in detection of disease and health status monitoring [19].

Several interesting evolutions have been made in the domain of biomarker research in ASD that possess the promise of spanning basic and clinical science in the future. Usually analyzed biomarkers of neuro developmental disorder include genetic, inflammatory, metabolic markers together with markers of mitochondrial dysfunction and oxidative stress. [20]. As of late, an economical and effective model for diagnosis of ASD arose in the field of genetics where microRNA analysis indicated encouraging outcomes in discerning ASD children from their typically developing peers [21]. Genetics investigations can be accomplished on samples of different origins, but for the sake of diagnosis, these sources are mostly saliva, blood or buccal swabs [22]. Noncoding RNA play a pivotal role in regulating structure of chromatin and gene expression of ASD patients[23]. MicroRNAs are small, regulatory noncoding regions of RNA that modulate expression of gene through posttranscriptional mechanisms by inhibition or degradation of specific mRNA

TABLE 1.3: Applications of Biomarkers and Related Examples [12].

Applications of Biomarkers	**Examples**
Biomarker is used as a diagnostic tool for the recognition of disease or abnormality	High glucose concentration in blood for the diagnosis of diabetes mellitus
For the exposing disease	carcinoembryonic antigen-125 measurement for various types of cancers
Use as a tool for classification of extent of disease	For the diagnosis of degree of tumor growth Prostate specific antigen concentrations in blood are used
For the specification of disease prognosis	In case of certain cancer tumor shrinkage anatomic measurement is used
For the prophecy and observing of clinical response to an intervention	For determination of heart diseases risk ,cholesterol concentrations in blood is used

targets. miRNAs have important role in physiological and pathological processes. Furthermore, miRNAs are more stable than mRNAs and also barely susceptible to slight differences in processing of samples. The above mentioned attributes make them remarkable biomarker candidates. Effectively and easily accessible sources of body fluids such as serum, blood, saliva or urine would provide an ideal source to miRNA biomarkers [1].

The perceptions about the miRNAs roles in CNS development such as cell differentiation and proliferation, apoptosis, synaptogeneis, and synaptic plasticity may be the significant factors to elucidate this unique biomarker for prognosis and diagnostic of disorders, along with the findings of up to date targets for drug improvement and development for therapeutic applications [24]. miRNAs are 70% expressed in the CNS and regulate almost two third of human mRNAs[25]. According to a recent study of World Health Organization (WHO) 10-20% of the offsprings and adolescents suffer with mental disorders all over the world [26], and even mild mental issues in childhood can prompt enormous problems as an adult. These issues escalate interest in child psychopathology, globally [24]. Moreover, it is tremendously tough and intuitive to diagnose psychological disorders, especially

for children because it predominantly counts on psychometric assessments. Thus, molecular evidence can play a significant role in diagnosis and becomes crucial and interesting. Biomarkers are specified candidates of biomolecules, consider the biochemical and physiological condition of an organism and assist as standard or indicators for pharmacological spontaneous reaction.

miRNAs have already come into applicable as beneficial clinical biomarkers for the prognosis and diagnosis of specific disease because of their disease specificity, accessibility and upraised stability [27].

Fluctuations in expression of miRNA patterns have been identified in the brain, saliva, blood and olfactory precursor's cells of ASD patients. Till now there are various identified miRNAs which show potential involvements in Autism spectrum disorder [28]. miRNAs are strongly associated with pathogenesis of ASD according to studies [21][29].

1.1 Problem Statement

There is no reliable blood test for the early diagnosis of Autism Spectrum disorder (ASD). Mostly ASD is identified on the base of behavioral assessments, due to which diagnosis of ASD is not possible before the age of two years when ASD child start showing symptoms.

1.2 Proposed Solution

There is a need of biomarker for the early diagnosis of ASD.

1.3 Aim of Study

As there is no standard biochemical or a molecular diagnostic criterion is available for ASD therefore, discovery of efficient diagnostic biomarker such as miRNA could be explored to evaluate their potentials as diagnostic biomarkers for ASD.

1.4 Objectives

This research mainly focuses on:

1. Selection of miRNAs which are involved inASD.
2. Prediction of target gene based on selected miRNA.
3. Functional analysis of target gene and miRNA.

1.5 Scope

Bioinformatics, in general, contributes through prediction of therapeutic targets which ultimately reduce manual efforts and cost of experimentation. So, in this study, we will contribute towards biomarker identification against ASD by predicting efficient miRNA for early diagnosis of ASD. The promising biomarker can be tested in experimental laboratory that can ultimately result in commercial product in future.

Chapter 2

Literature Review

2.1 Background

ASD is a heterogeneous neurodevelopment disorder with early childhood onset and may persist for life time. It involves lack of social interaction, deficits in socio-emotional reciprocity along with impaired verbal and nonverbal communication skills. A person suffering from ASD is unable to maintain, understand or develop relationships. Recently, it has been reported by the Centers for Disease Control and Prevention that 1/54 infants of age 8 in the United States in 2016 was suffered with ASD with males to females ratio of 4.3:1[30]. Global prevalence of ASD is increasingly recognized as 1% [31]. The worldwide rate of ASD is higher in males than females [32]. Autistic persons exhibit uncertain problematic actions, such as self injury, attacking, resistance to orders, unable to express own feelings and understand others, give unrelated answers to questions and failure of normal verbal and nonverbal conversation. It is difficult for ASD individuals to acquire the same education levels as their peers, or live independently. ASD is usually comorbid with sleep-wake disorders, social-anxiety, hyperactivity and obsessive compulsive disorders. Majorly, polygenic factors involving several different loci and gene-environmental interactions are involved in the onset of ASD. Different scientists gave different hypothesis regarding the causes of ASD. According to Hallmayer et al the ASD is caused more by environmental factors (58%) and

less by genetic effects (38%). In contrary to this, Sandier et al has said that Autism is increased with genetic disorder and there is no role of environmental factors [33]. DSM-5 is the only major psychological criteria used to diagnose ASD. The DSM-5 criteria have been used to accurately recognize ASD in toddlers and those with lenient or mild symptoms. In this criteria core symptoms are divided into two main domains such as social communication, and restrictive and repetitive behaviors[11]. Recent studies report that clinicians and researchers have shown uneasiness concerns related to the impact of new DSM-5 criteria because the complex as well as strict nature of criteria is not up to the mark or suitable for the early diagnosis of ASD[12]. These complexities encourage the development of biomarkers identification for early diagnoses and to predict treatment response in patients [34].

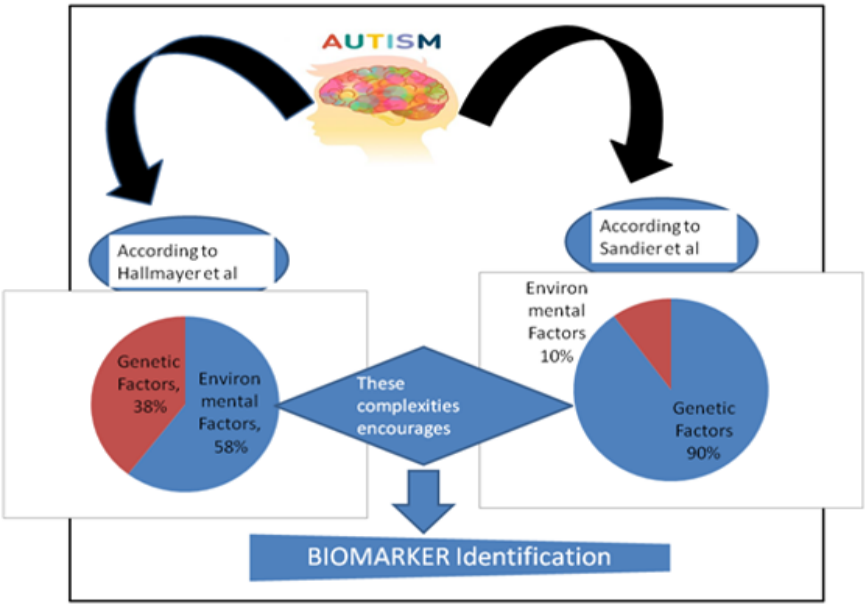

FIGURE 2.1: Flowchart Explaining Complexities which Encourage Biomarker Identification Technique

2.2 Prevalence of ASD

In current years, there has been a substantial increase in prevalence of ASD globally. It is now essential for policy development, nationally or locally, to understand the patterns of prevalence to make effectively plans. Moreover, in prevalence rates

among states and even among countries there are significant geographical differences which play their role[30].

2.2.1 Worldwide Prevalence of ASD

Literature survey proclaims the estimated prevalence among 11 locations was 1 in 54 children with age 8 years[35]. The most recent studies reported that ASD prevalence increases commonly such as 24.7% in adolescents and children in US and 11.5% in children with age 7-9 years in Italy[36]. ASD was more common in boys (23.6 per 1000) than in girls (5.3 per 1000) [25]. It has been reported in several studies that prevalence of autism in Bangladesh was 0.2% [37], 0.84% [38] and 0.15% [38]. In India reported prevalence was 0.09% and 0.23% [38]. Collective prevalence of infant or childhood autism was 3.923% in mainland China which is lower than fully developed countries[39]. In Arab Gulf countries the prevalence of ASD ranged from 0.14% to 2.9%[40]. In Pakistan no specific and reliable data is so far generated[41]. In Nepal no authentic estimated prevalence is measured due to lack of awareness in people and poor diagnosis [29].

Table 2.1: Prevalence Rate In Different Countries[30]

Country	**Age**	**Prevalence rate**	**Research**
USA	08 years old	1 in 54	Maenner et al. (2020)
CA,USA	22-Mar	1 in 50	Cardinal and Fraumeni-McBride (2017)
Eindhoven, The Netherlands	14-Apr	1in 44	Roelfsema et al. (2012)
Sweden	14-Mar	1 in 141	Sandin et al. (2014)
UK	16-May	1 in 111	Green et al. (2004)
Cambridgeshire UK	9-May	1 in 64	Baron-Cohen et al. (2009)
Toyota city, Japan	8-May	1 in 55	Kawamura et al. (2008)
Australia	0-05	1 in 455	Williams et al. (2008)
Canada	4-Feb	1 in 159	Quellette-Kunts et al. (2012)
Denmark	5.8	1 in 135	Parner et al. (2008)
Taiwan	5-Mar	1 in 621	Lai et al. (2012)

2.2.2 Prevalence of ASD in Pakistan

Pakistan is sixth most populous nation in the world with population of more than 181 million[42]. Almost 38% of population is under the age of 15. Although considerable improvements have been noticed in pediatric health department in last few decades, but still there is a lack of efforts and advancements in areas of child mental health and learning disabilities. Lack of awareness is the major cause of no reliable epidemiological data in Pakistan. Very few epidemiological studies are so far conducted in Pakistan[43].

TABLE 2.2: Prevalence Rate of ASD In Different Regions of Pakistan [43]

Region (Hospital (Based Data)	**Size of Sample**	**Total Number of Diagnosed Autistic Patients**	**Male to Female Ratio**	**Research**
Karachi	290	7 (2.4%)	———	Syed et al. (2007)
Rawalpindi	169	9(5.3%)	1:01	Tareen et al. (2009)
Karachi	200	9(4.5%)	2:01	Sarwar et al. (2009)
Lahore	1000	32(3.2%)	2:01	Imran et al. (2012)

2.2.3 Prevalence of ASD and Gender Differences

Studies showed that rate of ASD are much higher in males than in females with the ratio of 4:1[44]. Due to this striking and persistent feature of ASD of being more commonly diagnosed in males than females gave rise to leading proposal about the etiology and nature of ASD, such as the female protective effect[45], female autism phenotype theories and extreme male brain[46]. According to meta analysis conducted by Loomes et al 2017, there are certain factors which are responsible for this higher ratio in males; they argued that actual gender gap is less than this. One of the major arguments in their study is that the characteristics of autism in males and females are different and diagnostic bias is also observed [44].

2.3 Pathophysiology of ASD

Impairments in neural mechanisms observed in ASD remain mysterious, though contemporary development in genetics e.g. anatomical and physiological imaging technology, gross anatomy investigations, and molecular biology has furnished deep understanding. Gastrointestinal symptoms have also been associated with more frequent challenging behaviors among children with ASD, The etiology of ASD is not focused on genetic factors only but it also greatly depends on number of environmental factors and associated conditions such as immune imbalance and gastrointestinal activity. Redox sensitive metabolism, mitochondrial dysfunction, and carbon metabolism flaws have also been revealed in few subsets of ASD cases. Involvement of gut microbiota is also a major source of causing ASD. In addition both postmortem and neuroimaging studies and animal model of ASD manifest abnormalities in different regions of brain like cerebellum, hippocampus, frontal cortex, amygloid nucleus and cerebello thalamus pathways [47].

2.3.1 Environmental Factors

Environmental factors can influence the quantity and quality of gene expression without altering the sequence of DNA through epigenetics, by involving the changes in histone proteins or DNA methylation [48]. The correlation between epigenetic mechanisms can generate different variety of cell types during development and preserve the expression profiles of cell types throughout life [49]. Parental age has been highly correlated with increased autism risk in many studies [50][51][52]. Many studies identified that parents with more than age 34 are more responsible for reproducing offspring with increased risk of ASD [53][50]. Physiological and chemical agents are major environmental factors involved in causation of ASD[54]. Dietary and social factors are also needed to be investigated. Old parental age, low conception weight, premature birth, and neonatal jaundice is highly associated risk factors associated with environmental agents. Furthermore, pregnancy difficulties are also reliably shown up to be connected with the ASD. Traffic related air pollutants are highly related with ASD emergence. Maternal status is also linked

with the production of ASD[55]. Prenatal exposure to maternal tobacco smoke is majorly associated with methylation of DNA[49].

Table 2.3: Findings Related to Causation of ASD

Findings	**References**
Heavy Metals	
Study support strong association between heavy metals and association of ASD.	[56]
Both negative and positive associations in lead and manganese levels and ASD.	[57]
Lead	
Correlation of elevated lead level with decreased IQ levels.	[58]
Lead concentration in blood inversely and significantly related to IQ level.	[59]
Methyl Mercury	
Association with attention, language and memory.	[60]
Neurobehavioral deficits caused by prenatal methyl mercury exposure.	[61]
Prenatal exposure associated with cognitive deficits.	[62]
Air Pollution	
Exposure to air pollution during childhood or gestational period strongly related to causation of ASD.	[56]
Evidence of positive association of PM2.5, weak evidence of NO2, and little evidence of PM10 and ozone.	[63]
Endocrine disruptors	
Prenatal exposure to endocrine disruptors is associated with adverse neurobehavior.	[64]
Pthalates	
Prenatal exposure to phthalates strongly associated with behavior domains.	[65]
Prenatal exposure to Di-n-butyl phthalate, Benzyl butyl phthalate and Diisobutyl phthalate may associate with adverse effects of child motor, mental and behavioral development.	[52]
Bisphenol A	
Bisphenol A exposure during gestation may reduce TSH in girls.	[66]
Exposure of BPA causes alterations in gene expression in specific regions of brain.	[66]
Perfluoroalkyl substances	
PFOs induced in 10 days old mice, which result in impaired behavioral activities when mice were 2 and 4 months old.	[67]

2.3.2 Gut Microbiota Factors

It has been reported in many studies that specific GI phenotype is involved in ASD which is caused by increased gut permeability and abnormal immune functions in gut. Prevalence of gastrointestinal symptoms is four times higher in autistic children than typical developed children [68].

Thousands of distinct species of microbes and additional 15000 kinds of bacteria are involved in composition of gut microbiota. The size of bacteria involved is equal to 1 kg. Gut microbiota is considered as first defense line for protection of gastrointestinal tract [69].

It has been reported that gut microbiota is involved in alteration of immune neural and endocrine pathways and due to this it is called as gut brain axis [70][71].

Few researches in past reported that imbalance of microbes (dysbiosis) is highly responsible for pathogenesis of ASD [72][73]. The role of gut microbiota in the etiology of ASD has been explained through several studies by using different experimental approaches in animal models such as comparison of gut microbiota, and observation of behavior changes by examining gut micro biota.

Gut microbiota play an important role in the body, and imbalances in their structure and diversity can lead to a variety of diseases. The host promotes a healthy microbiota by releasing both particular and nonspecific factors including antimicrobial peptides, mucus, and immunoglobulin.

It has been reported that large figure of species of Clostridium genus is involved in autistic children fecal samples [74]. Bacteroidetes and Firmicutes phyla are highly imbalanced and number of Bacteroidetes is increased in ASD.

Different gut microbes colonies such as Lactobacillus, Sutterella, Prevotella, Ruminococcusgener aand Alcaligenaceae are highly disturbed in children with ASD [75].

Bolt in 1998 observed that eloquent percentage of persons with ASD used antibiotics extensively in history. Due to high usage of antibiotics the intestinal tract is disrupted [28].

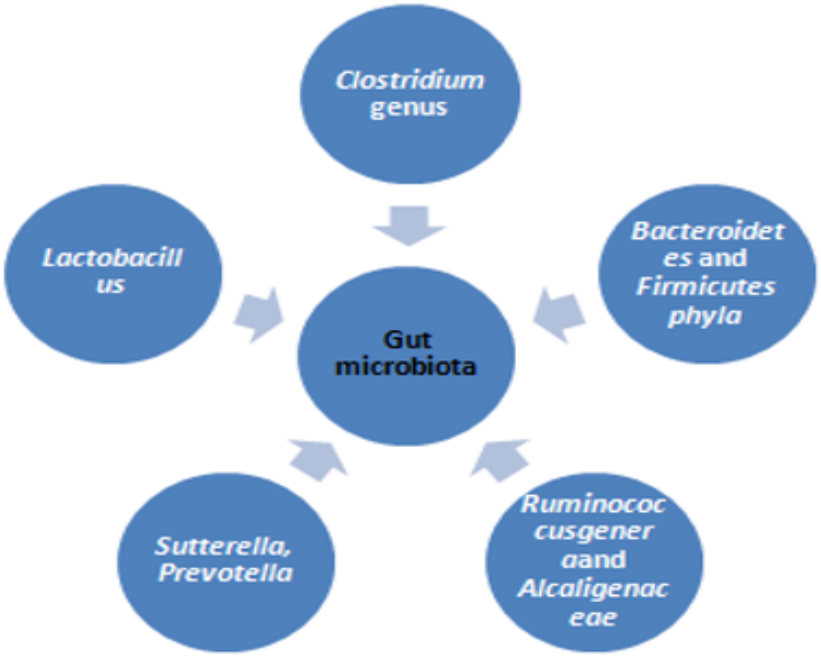

FIGURE 2.2: Major Gut Microbiota Modifications Involved in Etiology of ASD[17]

2.3.3 Genetic Factors

Over the last few years, advancements in technology have encouraged the relevant DNA variants identification and are highly correlated with ASD. Large number of rare single nucleotide variants (SNVs) including very small insertions/deletions mutations related with ASD is easily identified by next-generation sequencing (NGS), whole genome sequencing (WGS) and whole exom sequencing (WES). Moreover, family based sequencing has facilitated the removal of causal relationship of de novo and inherited variants, such that it has now been revealed that hundreds of genetic variations affect a wide range of molecular functions related to ASD. Notably, these genes are not distributed in random manner but present in functionally relevant biological processes such as transcriptional regulation in brain and synapse development[76]. Many genetic factors including gene mutations contributes to ASD causing abnormal brain development [33]. The internal symptoms of ASD are immune dysregulation, abnormal level of cytokines and growth factors [4]. Epilepsy is one of the major disorder associated with genetic factor of ASD [77]. Extra suggested components need aid expanded oxidative stress,

mitochondrial dysfunction, abnormalities clinched alongside mind serotonin, abnormal white is concerned connectivity modified synapses coming about because of hereditary changes, for example, transforms clinched alongside ERK pathway [78]. An assortment about morphologic and practical progressions need have been shown in the brain for kids alternately mature people for ASD [79].

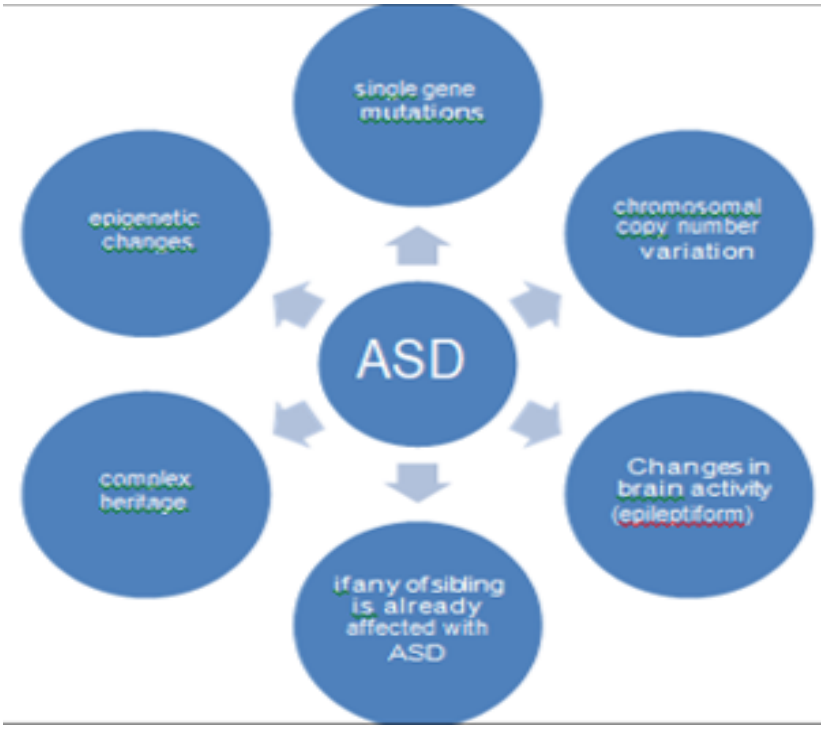

FIGURE 2.3: Genetic Factors Involved in Etiology of ASD [80]

2.3.3.1 De Novo Variants

One of the major genetic finding correlated with ASD is the identification of de novo variants (DNVs). DNVs are basically DNA variants which are occupied in the prenatal germ line during meiosis. DNVs have wide range of genomic alterations similarly as inherited variant comprises. Genomic alterations include insertions, deletions, copy number variants (CNVs) and single nucleotide polymorphism (SNPs)[81][82][83][84][85][86]. DNVs have greater genetic effect in sporadic ASD. Large scale screening studies have been used to identify CNVs and de novo variants associated with ASD[82]. Various loci were distinguished by denovo CNVs such as 1q21.1, 7q11.23, 15q11-13, 16p11.2, 17q12 and 22q11.2.

The above mentioned CNVs are larger deletions and duplications that surround multiple genes and regulatory regions. Important synaptic genes such as NLGN3, NRXN1, SHANK3, SHANK2 and SYNGAP1 were shown highly affected by many of these denovo CNVs. These genes are involved in synaptic dysfunctions or perturbation of synapse development underlining the pathophysiology of ASD[76].

2.3.3.2 Rare Inherited Variants

Rare inherited variants accounts for the considerable genetic conversions in the human genome in contrast to denovo variants. Common and rare variants, both of them are involved in the etiology of ASD. Rare variants have higher frequency of involvement in development of ASD than common. WES studies have greatly identified rare inherited variants interlinked with ASD[76].

2.3.3.3 Common Variant Associated with ASD

According to studies almost $> 1\%$ of population has affected by common variants corresponding with ASD. Individual common variants have little consequence or benign effect on disease, unlike rare variants [87]. In case of ASD, common variants caused about 40% of genetic risk. Researchers found a variant (rs17225178) related to ARNT2 gene which is involved in important transcription factor [88]. Primarily ARNT2 gene is highly known for regulation of cellular responses to hypoxia with HIF1A in CNS [89]. rs789859 in the 3q29 region is a common variant related to learning difficulties and also associated with ASD [76].

2.4 Genetic Heterogeneity of ASD

ASD is caused by multiple genes. Some of the loci identified through OMIM which may enroll in phenotype are discussed in Table 2.4. The identified loci mapped in specified chromosome.

Table 2.4: Loci Identified on Mapped Chromosomes Involved in Onset of ASD Phenotype

Loci Identified	Mapped Chromosome	Description	Reference
AUTS1	chromosome 7q22	maximum multipoint lod score of 2.15 at marker D7S477	International molecular genetics consortium(2011)
AUTS3	chromosome 13q14	de novo deletion of 13q14-q22	Ritvo et al.(1988), Steele et al. (2001)
AUTS4	chromosome 15q11	interstitial duplication of 15q11-q13	Bundey et al. (1994),Clayton-Smithet al. (1993), Filipek et al. (2003)
AUTS5	chromosome 2q	maximum multipoint heterogeneity lod score (hlod) of 1.96 and a maximum multipoint nonparametric linkage (NPL) score of 2.39 on 2q	Buxbaum et al. (2001)
AUTS6	chromosome 17q11	multipoint lod score of 2.34	International Molecular Genetic Study of Autism Consortium(2001)(IMGSAC)

Continued Table 2.4: Loci Identified on Mapped Chromosomes Involved in Onset of ASD Phenotype

Loci Identified	Mapped Chromosome	Description	Reference
AUTS7	chromosome 17q21	maximum lod score of 4.1 at marker D17S2180	Cantor et al. (2005)
AUTS8	chromosome 3q25- q27	maximum 2-point lod score of 3.16 with marker D3S3037	Auranen et al. (2002)
AUTS9	chromosome 7q31	a novel gene, called RAY1 (ST7; 600833), on 7q31 was interrupted by the translocation breakpoint, suggesting that coding mutations in the RAY1 gene are unlikely to be involved in the etiology of autism.	Vincent et al. (2000)
AUTS10	chromosome 7q36	strongest QTL evidence for the age at first word on 7q35-q36	Alarcon et al. (2002)
AUTS11	chromosome 1q41	multipoint lod score of 3.06 and 2-point nonparametric lod score of 3.21 at D1S1656	Buxbaum et al. (2004)(Maussion et al., 2008)

Continued Table 2.4: Loci Identified on Mapped Chromosomes Involved in Onset of ASD Phenotype

Loci Identified	Mapped Chromosome	Description	Reference
AUTS12	chromosome 21p13- q11	nonparametric lod score of 3.0 near marker D21S1437; maximum multipoint lod score of 3.4 under a dominant mode of inheritance	Molloy et al. (2005)
AUTS13	chromosome 12q14	multipoint parametric lod score of 3.02 at SNP rs1445442under a recessive model	Ma et al. (2007)
AUTS14A	chromosome 16p11.2	identified 27 individuals with a 16p11.2 deletion and 18 with a 16p11.2 duplication, most commonly for developmental delay and mental retardation	Shinawi et al. (2010)
AUTS14B	chromosome 16p11.2	Identified 18 individuals with a 16p11.2 duplication	Shinawi et al. (2010)

Continued Table 2.4: Loci Identified on Mapped Chromosomes Involved in Onset of ASD Phenotype

Loci Identified	Mapped Chromosome	Description	Reference
AUTS15	chromosome 7q35- q36	associated with mutation in the CNTNAP2 gene	Alarcon et al. (2002)
AUTS16	chromosome 3q24	associated with mutation in the SLC9A9 gene	Morrow et al. (2008)
AUTS17	chromosome 11q13	associated with mutation in the SHANK2 gene	Berkel et al. (2010)
AUTS18	chromosome 14q11.2	associated with mutation in the CHD8 gene	O'Roak et al. (2012)

2.5 Medical Co Morbidities Associated with ASD

Autism spectrum disorder incorporates conditions that were formerly viewed as isolated such as autism, childhood disintegrative disorders, Aspergers's syndrome and an unidentified character of pervasive developmental disorder[31]. The severity of autistic characteristics along with the occurrence of co morbid illnesses which incorporate anxiety, intellectual incapacity, epilepsy, and gastrointestinal disorders substantially vary among individuals of autism [5][6][7][8]. For the better understanding of basic biological dysfunction identification of ASD, associated co morbidities are important to be considered so that it can lead toward treatment plan[90].

2.5.1 Seizures and Epilepsy

The prevalence of seizure and epilepsy is much higher in autistic individuals as compared to typically developing individuals. Prevalence rate of seizure is 20%-35% in autistic adults and 7%-14% estimated in autistic children [91]. Mortality, severe ASD symptoms and intellectual disability is highly associated with epilepsy, more importantly if it continues into adulthood. The major cause of epilepsy is more frequently related to metabolic or genetic abnormalities. It has been reported in many studies that children with ASD and epilepsy have intellectual impairment, higher socialization problems and hyperactivity as compared to children having ASD and SEDs [90].

2.5.2 Neurotransmitter Disorders

Multiple neurotransmitter disorders have been reported to associate with ASD. These disorders include certain type of neurotransmitters such as monoamine e.g. dopamine, serotonin and norepinephrine, amino acid e.g. glutamate, gamma-aminobutyric acid (GABA), and cholinergic e.g. acetylcholine. The basic etiology of these type of disorders are not exactly known but few studies using animal models of ASD have noticed genetic mutations which disrupt neurotransmitters. A

neurotransmitter, oxytocin is recognized to play a role in ASD specially associated with social impairment [90].

2.5.3 Sleep Disorders

The prevalence of sleep abnormalities are higher in ASD estimated 40% to 80% as compared to typically developing individuals [91]. Sleep disorders related to ASD are common including prolonged sleep onset latency, awakening at night, less sleep duration, insomnia and daytime sleepiness. Impaired social interaction, communication, anxiety and poor quality of life have been associated with sleep disorders.

TABLE 2.5: Melatonin Metabolites Associated with Sleep Disorder in ASD [90]

Melatonin Metabolites Associated with Sleep Disorders in ASD
Endogeneous neurohormone which is known for regulation of good sleep is highly fluctuated in ASD.
6-Sulfatoxymelatonin is the prominent melatonin metabolite which is diminish in some cases of ASD causing decrease in melatonin production and inversely related with deep sleep.
N-acetylserotonin, the melatonin metabolite is elevated in ASD and has inverse correlation with amount of melatonin.

2.5.4 Gastrointestinal Disorders

It has been reported in many studies that about 91% of autistic children can be affected by wide range of gastrointestinal (GI) symptoms. According to recent studies ASD infants were more prone to have diarrhea, and food allergy, constipation. Some of unique and distinctive abnormalities in GI functions have been linked with ASD, incorporating nonspecific inflammation and abnormal transportation of carbohydrates across enterocytes. Evidence suggests that enteric biome that inhabit the human digestive tract can highly affect metabolic and immune activities, regulate gene expression and involve in development of behavior and brain

.A study on mouse model showed the importance of microbiome associated with ASD. The researchers highlighted the fluctuations in the enteric biome in this study [90].

2.5.5 Metabolic Disorders

Metabolic disorders are highly associated with ASD according to several studies. Different type of metabolic disorders that seem to affect a small remarkable portion of ASD. Actual prevalence of metabolic disorders in ASD is still unknown[90].

Table 2.6: Metabolism Types Which are Involved in ASD[90].

Metabolism	**Findings**
Folate Metabolism	Polymorphism that is involved in causation of decrease in 5methyltetrahydrofolate and impairs folate transportation to neurons is highly associated with ASD.
Cobalamin metabolism	A study reported that cerebral cobalamin deficits are highly associated with ASD.
Tetrahydrobiopterin metabolism	According to study, concentrations of cerebrospinal BH4 are reduced in case of ASD.
Carnitine metabolism	It has been reported that a gene (TMLHE) which encodes for the enzyme of carnatine biosynthesis pathway found defected in 7 autistic children.
Redox metabolism	Several studies evident that abnormal redox metabolism can be associated with ASD.
Mitochondrial metabolism	One of most prevalent disorder related to ASD is mitochondrial metabolism. Mitochondrial metabolism in ASD associated it with developmental delay and epilepsy.
Cholesterol metabolism	It has been reported that autistic children have lower level of cholesterol as compared to typically developed children.

2.5.6 Immune Disorders

Several studies have been reported that the immune system has a participating role in ASD. According to studies, when ancient animal models of ASD came

across with prenatal exposure to lipopolysaccharide, a maternal immune response was induced.

TABLE 2.7: List of Immune Disorders Associated with ASD[90].

Immune disorders	**Findings**
Auto antibodies	Various studies reported the biomarkers of autoimmunity associated with ASD. Antinuclear, Antinucleosome antibodies and C-reactive protein has shown to be fluctuated in ASD.
Hypogammalobulinemia	It has been reported that both increase and decrease levels of hypogammaglobulinemia are involved in ASD.
Neuroinflammation	Studies suggest the close accessibility of microglia with neurons in the autistic children brain.
Cytokine abnormalities	Abnormal cytokine concentrations in the blood, brain and cerebrospinal fluid of autistic children has been reported.

2.6 Diagnosis and Treatment

There is no efficient diagnostic biological or blood test available for ASD. ASD cannot be diagnosing before the 2 years of age and it is becoming serious issue day by day. The two diagnostic criteria are based on behavior and communication which are commonly used for determining the presence of ASD. It is reported in certain studies that currently diagnosis is based on two criteria which include observing the patient's behavior or his impairment communication skills [78]. Delayed general development or defective linguistic skills are considered common symptoms for early diagnosis [92]. Different measuring scales or questioners are used for diagnosing behavioral and communication impairment; few of them are Friendship

Qualities Scale (FQS), Quality of Socialization Questionnaire (QSQ), Social Interaction Anxiety Scale (SIAS), Social Skills Rating System (SSRS), Kaufman Brief Intelligence Test-Second Edition (KBIT-2) [30]. It is also reported innovative developments have enabled robots to satisfy an assortment of human-like functions and ensuing acceptance from claiming intercessions to people for mental imbalance range confusion (ASD) [93]. Few studies recommend face book as a useful tool or diagnostic instrument to understand the mental capability of ASD patients [33]. Despite of all above mentioned diagnostic criteria there is still no biological test for early diagnosis , so that the ratio of ASD can be controlled at the onset of disorder. So there is a need to produce certain biological diagnostic tests so that detection of ASD can be occurred at proper time earlier.

TABLE 2.8: Diagnostics Criteria Used for Identification of ASD [47].

Diagnostic Criteria	
Criteria A	**Criteria B**
Repetitive speech/motor movements and use of objects.	Socio-emotional reciprocity deficits.
Rigid constancy to routine.	Nonverbal communication deficits.
Incredible sensory interests.	Maintaining developing and understanding relationships.
Extremely suppressed, obsessed interests with abnormal Intensity.	Decreased interest in social interaction.

2.7 Biomarkers

Biological markers about disease are defined as biological variables related together with a disorder yet measureable between a patients or within his/her biomaterials using quantitative procedures. Biomarkers are the molecules e.g. genes, proteins, metabolites in fluids such as serum, urine and saliva which helps in detecting the normal processes or indicating the presence of specific diseases [94]. Biomarkers not only depend on genotype but also on phenotype of the organism [95]. Biomarkers are very helpful for clinical purposes as they provide: chance estimates at birth in

baby siblings of teenagers already diagnosed with autism. Biomarkers can easily diagnose diseases in the early age i.e. between 12 and 30 months, they can predict developmental changes and biomarkers can identify the organism which is at high risk of bad reaction to psychoactive drugs.

Endophenotypes includes a lot of biomarkers which helps in defining biologically homogeneous groups of ASD patients and it is also helpful in understanding the purpose and psychological changes that occur because of this disease.

The genetic and environmental factors that cause neuro-developmental disorders are identified by biomarkers and these organisms are treated by many molecular therapies [34].

2.7.1 Types of Biomarkers

There are three major types of biomarkers used for detection of disease and clinical purposes.

2.7.1.1 Diagnostic Biomarkers

These biomarkers are specifically used for singling out of disease. The identification of onset of disease to better cure at right time is the main goal of diagnosing biomarkers.

For example "human chronic gonadotrophin" (hCG) is a biomarker which is present in the urine for the detection of pregnancy [96].

2.7.1.2 Prognostic Biomarkers

These biomarkers predict about a conceivable growth result (e.g. illness repeat, infection movement, and passing) free of treatment got. For example,

Anti-cyclic Citrullinated Protein Antibodies (ACPA) or Rheumatoid Factor (RF) are the auto antibodies which are present is about two third of Rheumatoid Arthritis individuals [97][98]. These two auto antibodies act as prognostic biomarker for identifying the progression of certain disease [99][100].

2.7.1.3 Theranostic Biomarkers

These biomarkers support treatment choice and could identify suitable treatment for an individual according to individual's current condition or predict drug response [99]. Drug identification targets are identified by these biomarkers as well. For example propagation of chronic inflammatory processes involved different signaling pathways which act as potential source of theranostic biomarkers for generating response to anti-inflammatory therapies e.g. anti TNF [99][101]. Various applications of biomarkers are summarized in table 2.8. Computational biology has significant role in the new biomarkers intervention. Some other types of biomarkers are: Morphological biomarkers, Neurophysiological biomarkers, Hormonal biomarkers and Immunological biomarkers.

Table 2.9: Application of Biomarkers in Various Diseases

Biomarker	**Objective Use**	**Examples**
Diagnostic	for disease identification	"humanchronicgonadotrophin" (hCG) in the urine for the detection of pregnancy.
Prognostic	for predicted outcome disease progression	(ACPA) or (RF) is the auto antibodies present in Rheumatoid Arthritis.
Theranostic	for the identification of appropriate treatment	P38 signaling pathway is involved in apoptosis, cytokine production and inflammation.
Biochemical	present in blood, so that easily detectable	Serotonin recorded in blood of ASD individuals.
Morphological	for elucidation of cellular and/or tissue damage	Head Circumference measured in ASD individuals.
Neurophysiological	For clinical imaging and neuroimaging	Neuro imaging parameters in ASD.
Hormonal	for detection of hormonal chemicals imbalance	Melatonin played major role in seasonal heart rhythms.
Immunological	related with immune disabilities	Increased or decreased amount of T lymphocytes.

2.8 miRNA as a Biomarker

miRNAs are small non coding regions of RNA which are approximately made up of (18 -22) nucleotides, involved in the regulation of more than half of the genes in human cell. They suppress translation and initiate degradation of mRNAs when complementary binding occurs. This deregulation of the expression of miRNA causes disease [102]. Several studies revealed that fluctuations in miRNAs amount are common with the increase in age [116].

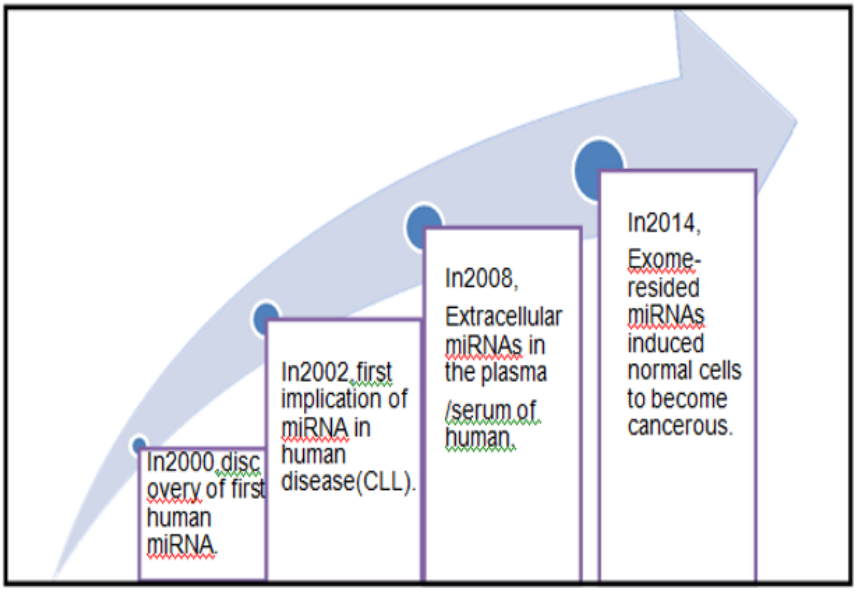

FIGURE 2.4: Timeline of the Important Discoveries About the Use of miRNAs as Bio-Markers

2.8.1 Mode of Action of miRNA

miRNAs regulation starts at transcription and post transcriptional levels. RNA polymerase II or RNA polymerase III transcribed miRNAs to form a loop like structured molecule which is called primay miRNA(pri-miRNA) which gets transcribed inside the nucleus. Then Drosha which is RNA III enzyme and the DGCR8/Pasha protein complex cleaves the pri-miRNA forming hairpin miRNA of approx 70 nucleotides. Now, Exportin-5 transports this hairpin miRNA in to cytoplasm [41]. Dicer which is another RNase III enzyme cleaves the pre-miRNA second time to form a duplex structure of miRNA of about approximately 22

neuclotides. This structure is then divided into two single strands of miRNA which binds to a protein named Agonauute-2 (Ago2) and is integrated into the RNA-induced silencing complex (RISC) structure which helps miRNA to find out complementary sites for binding of target mRNA where expression of gene will be inhibited [103][104].

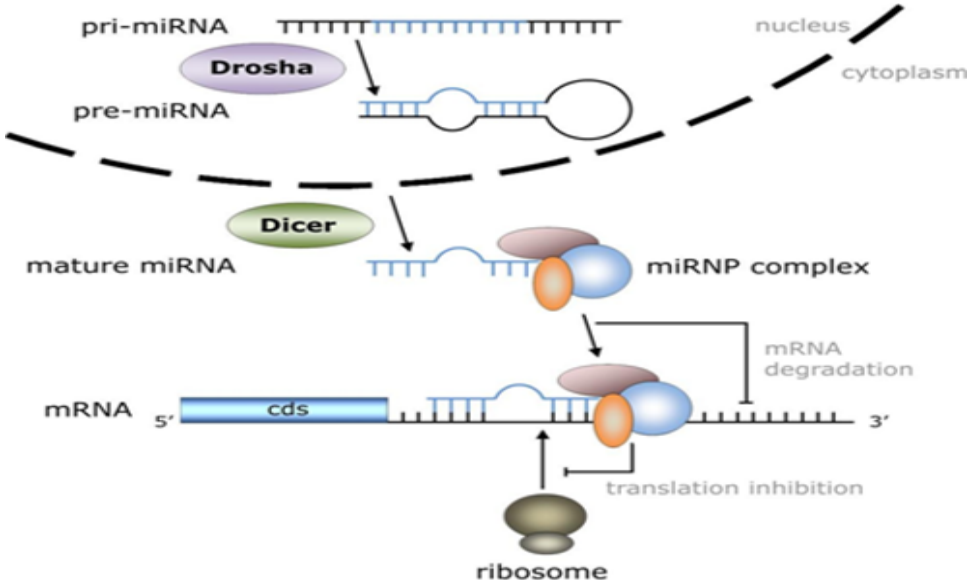

FIGURE 2.5: Biogenesis of miRNA Involved in Postranscriptional Level [61]

TABLE 2.10: miRNA Used as a Biomarker in Diseases [63].

Biomarkers	**Reference**
miRNAs as biomarkers for cancers	(Calin et al., 2004b)
Dysregulation of Cellular miRNAs in Viral Infections	(Jopling et al., 2005; Kutok and Wang, 2006; Chen et al., 2014a; Scaria et al., 2006; Murakami et al., 2009; Luna et al., 2015; Zhang et al., 2010a)
miRNAs in Nervous System Disorders	(Ling et al., 2010)
miRNA-based diagnostics in cardiovascular diseases	(Bernstein et al., 2003; Zampetaki and Mayr, 2012; Philippen et al., 2015)
miRNAS as auspicious biomarker in Diabetes Mellitus and metabolic disorders	(Lynn, 2009)
miRNA in many other diseases	(Stanczyk et al., 2008), Michael et al., 2010), (Zhang et al., 2010b), Ben-Dov et al., 2014).

Table 2.11: Description of Tools Used in Identification of miRNA for ASD

	Databases used for identification of miRNA			
Sr. No	**Databases**	**URL**	**Description**	**References**
1	miR2Disease Base	http://www.mir2disease.org/	It is a carefully organized database, provides a detailed comprehensive depository of miRNAs associated with various human diseases	Jiang Q., Wang Y., Hao Y., Juan L., Teng M., Zhang X., Li M.,Wang G., Liu Y., (2009)
2	PhenomiR 2.0	http://mips.helmholtz-muenchen.de/phenomir/main/showmir/6809?manorder=asc&sort=pm.mir.name	The PhenomiR database provides differentially regulated miRNA expression in diseases. It is manually curated database of experienced annotators.	Ruepp, A., Kowarsch, A., Schmidl, D., Buggenthin, F., Brauner, B., Dunger, I &Theis, F. J. (2010).
3	HMDD v2.0	http://210.73.221.6/ hmdd	The Human microRNA Disease Database is a curated database that has experimental approved for human microRNA and disease associations.	Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q. An analysis of human microRNA and disease associations. PLoS One. 2008. Li Y, Qiu C, Tu J, Geng B, Yang J, Jiang T, and CuiQ.
4	miRo‘2	http://microrna.osumc.edu/miro/	The miRò2 Database is developed and maintained at Prof. Croce's lab, Department of Molecular Virology, Immunology and Medical Genetics. It a manually curated database provides information of the miRNA.	Laganà, A., Forte, S., Giudice, A., Arena, M. R., Puglisi, P.L., Giugno, R & Ferro, A.(2009).

Databases Used for Target Genes Predictions				
1	TargetScan Humanv.7.1	http://www.targetscan.org/vert_71/	In TargetScan Human 7.1the algorithm used on basis of scoring of miRNA target gene.	AgarwalV, Bell GW, Nam J, Bartel DP (2015) Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, Baek D, Johnston WK, Russ C, Luo S, Babiarz JE, Blelloch R, Schroth GP, Nusbaum C, Bartel(2010)

Databases Used for Target Genes Predictions				
2	MicroT v4	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index	Diana microT is the gene prediction database in which the conserved and non-conserved regions of the miRNA elements are responsible for prediction using scoring techniques.	Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, FilippidisC,Dalamagas T, Hatzigeorgiou AG. (2013). T. Vergoulis, M. Alexakis, T. Dalamagas, M. Maragkakis, A. G. Hatzigeorgiou (2012) 628-633
3	miRDB	http://www.mirdb.org/cgi-bin/search.cgi	miRDB is an online database for miRNA target prediction. It uses the machine learning methods for the prediction of miRNA. On this base it gives the target scores for each Gene.	Nathan Wong and Xiaowei Wang (2015). Xiaowei Wang(2016)

Databases Used for Target Genes Predictions				
4	miRanda	http://34.236.212. 39/microrna/ home.do	miRanda is an open source which is used to find the targets predictions. It also has some scoring criteria.	Betel D, Koppal A, Agius P, Sander C, Leslie C. (2010)
5	PicTar	http://www.pictar.org/	PicTar database algorithm is for finding the targets of the miRNA which is based on the 3'UTR's predictions for particular sites. It also having the scores according to the sites conservations.	Creighton, C. J., Nagaraja, A. K., Hanash, S. M., Matzuk, M. M., &Gunaratne, P. H. (2008)

Database used for functional annotation of target genes				
1	DAVID v6.8	https://david.ncifcrf.gov/ tools.jsp	It is a database for Annotation, Visualization and Integrated Discovery. It has different sets of tools through which functional annotation is done to know about integrated pathways behind the genes.	Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. (2009)

Databases used for functional annotation of miRNA				
1	Diana mir-Path v.3	http://snf-515788.vm.okeanos.grnet.gr/	DIANA-mirPath is a miRNA pathway analysis webserver. It predicts the miRNA targets which is already provided by the DIANA-microT-CDs algorithm. The interactions are combined which is result of merging and doing meta-analysis of these interactions.	Vlachos, Ioannis S., KonstantinosZaggan-as, Maria D. Paraskevopoulou, GeorgiosGeorgakilas, DimitraKaragkouni, ThanasisVergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou. -2015
2	miRDB	http://www.mirdb.org/cgi-bin/search.cgi	miRDB is an online database for miRNA target prediction and functional annotation .	Nathan Wong and Xiaowei Wang (2015). Xiaowei Wang (2016)

Databases used for network and pathway enrichment				
1	Diana mirPath v.3	http://snf-515788.vm.okeanos.grnet.gr/	DIANA-mirPath is a miRNA pathway analysis webserver. It predicts the miRNA targets which are already provided by the DIANA-microT-CDs algorithm. The interactions are combined which is result of merging and doing meta-analysis of these interactions.	Vlachos, Ioannis S., KonstantinosZagganas, Maria D. Paraskevopoulou, GeorgiosGeorgakilas, DimitraKaragkouni, ThanasisVergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou. -2015
2	miRDB	http://www.mirdb.org/cgi-bin/ search.cgi	miRDB is an online database for miRNA target prediction and functional annotation. It uses the machine learning methods for the prediction of miRNA. On this base it gives the target scores for each gene.	Nathan Wong and Xiaowei Wang (2015). Xiaowei Wang(2016)

2.9 Prediction of miRNA as a Biomarker in ASD

Post transcriptional mechanisms such as miRNAs modulate expression of gene without altering the genetic code. miRNAs are deregulated in children with ASD. In ASD patients, the miRNA patterns are modified in brain, blood and saliva. The potential of miRNA as biomarker is found in serum and saliva of ASD patients. In the serum miRNAs of 55 children, 3 miRNAs are identified (130_3p,) miR_181b_5p, and miR_320a). It has been reported that patterns of salivary miRNAs is related with the adaptive behavior of effected child [105].

Chapter 3

Materials and Methods

3.1 Methodology of Identification of Efficient Biomarker for Diagnosis of ASD

In the identification of efficient biomarker very first step was to select the microRNAs (miRNAs) which were experimentally validated to be involved in autism spectrum disorder (ASD) particularly present in blood, serum and plasma.

For these purpose three databases e.g. phenomiR2, HMDD and miR2disease were used for the detection of miRNAs involved in ASD.

Then the three different lists of miRNAs were retrieved from three different databases, combined and redundancy was removed. miRNAs that matched from all of three databases were kept in a single list.

A short listed miRNAs after redundancy removal were prioritized based on target gene list and statistical significance of target gene. Target prediction was performed for the selected miRNAs, indicating that which miRNA is involved with genes in these databases.

Moreover, duplicated genes were eliminated and the remaining genes were only kept if all of them showed their presence in three predicted algorithms, giving putative list of target genes. The prediction of target was done separately for

both upregulated and downregulated miRNAs. The known genes for comparison with candidate genes were obtained from Autism Informatics portal (AutDB).

The selected targeted genes were then compared against 991 known genes associated with ASD, which were acquired from the Autism Database (AutDB) to look over either any of predicted target genes were related to ASD or not.

Then, Functional Annotation was done for the entire gene list separately by using clustering tool available on DAVID (Version 6.7) (The Database for Annotation, Visualization and Integrated Discovery).

In this way, gene list was prioritized further. The shortened gene list was then put in to VENNY tool and compared to the list of 991 known genes. The overlapped genes were considered as remarkable and used for further study.

Then functions of up regulated and down regulated miRNAs were determined through Diana-mirPath. The liable functions for the down-regulated and up-regulated miRNAs were queried separately using default settings. Annotated functions of miRNAs and predicted target genes were then compared to observe if there were any overlaps.

The genes which were against the miRNA annotated were saved in .xlm files, and then compared with the known upregulated targeted genes, to find the predicted genes. After comparing through the venny tool, genes showed their functions per pathways.

For significant and efficiency of that pathways, a heatmap was generated after the pathways union. The STRING version 11 (Search Database for the Retrieval of Interacting Genes) is a web based tool which is greatly used for analyzing protein interactions (PPI), gene ontology (GO) annotations, and KEGG pathway analysis.

Hence, KEGG pathway analysis was done separately for downregulated and up-regulated lists of target genes. Up regulated and down regulated genes were then separately queried and mapped to STRING for gene network enrichment and KEGG pathway.

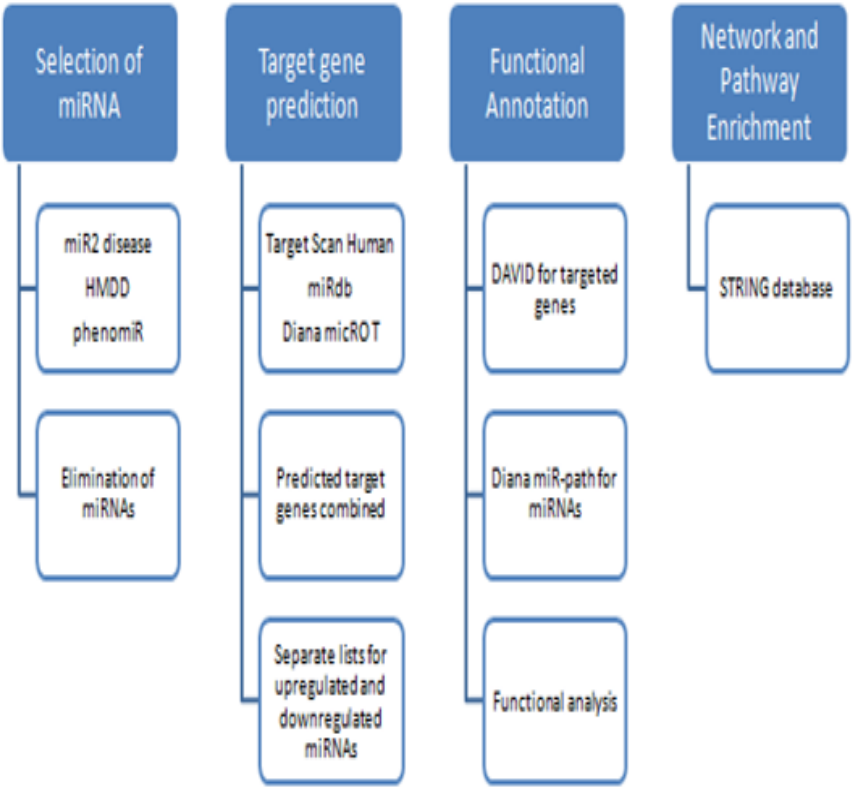

FIGURE 3.1: Methodology Used for Identification of miRNA as a Biomarker for Diagnosis of ASD

3.2 miRNA Dataset Selection

The microRNAs (miRNAs) which were experimentally validated, involved in autism spectrum disorder (ASD) particularly present in blood, serum and plasma, were selected from databases which are given in **Table 3.1**.

3.3 Target Gene Prediction

Target gene prediction was performed for the selected miRNAs, to indicate the impact and association of miRNA with these databases. The databases have different criteria for prediction of genes, mainly focuses on conserved regions. The databases used for target gene predictions are listed in **Table 3.2.**

TABLE 3.1: Database Available to Select miRNAs Involved in ASD

Sr.no	Databases	URL	Description	References
1	miR2Disease Base	http://www.mir2disease.org/	It is a carefully organized database, provides a detailed comprehensive depository of miRNAs associated with various human diseases.	Jiang Q., Wang Y., Hao Y., Juan L., Teng M., Zhang X., Li M., Wang G., Liu Y., (2009)
2	PhenomiR 2.0	http://mips.helmholtz-muenchen.de/phenomir/-main/showmir/6809?manorder=asc&sort=pm.mir.name	The PhenomiR database provides differentially regulated miRNA expression in diseases. It is manually curated database of experienced annotators.	Ruepp, A., Kowarsch, A., Schmidl, D., Buggenthin, F., Brauner, B., Dunger, I &Theis, F. J.(2010).
3	HMDD v2.0	http://210.73.221.6/hmdd	The Human microRNA Disease Database is a curated database that has experimental approved for human microRNA and disease associations.	Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q. An analysis of human microRNA and disease associations. PLoS One. 2008. Li Y, QiuC,Tu J, Geng B, Yang J, Jiang T, and Cui Q.

TABLE 3.2: Databases for Target Gene Predictions of Selected miRNAs

Databases	**URL**	**Description**	**References**
(1) Target Scan Human v.7.1	http://www.targetscan.org/vert_71/	In TargetScan Human 7.1 the algorithm used on basis of miRNA target gene scoring.	AgarwalV, Bell GW, Nam J, Bartel DP (2015) Chiang HR, Schoenfeld LW, Ruby JG, Auyeung VC, Spies N, Baek D, Johnston WK, Russ C, Luo S, Babiarz JE, Blelloch R, Schroth GP,Nusbaum C, Bartel (2010)
(2) Diana MicroT v.4	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index	Diana microT is the gene prediction database in which the conserved and non-conserved regions of the miRNA elements are responsible for prediction using scoring techniques.	ParaskevopoulouMD,GeorgakilasG, Kostoulas'N, VlachosIS,Vergoulis T, Reczko M, FilippidisC,Dalamagas T, Hatzigeorgiou AG.(2013). T. Vergoulis, M.Alexakis,T. Dalamagas,M. Maragkakis, A.G. Hatzigeorgiou (2012) 628-633
(3) miRDB	http://www.mirdb.org/cgi-bin/search.cgi	miRDB is an online database for miRNA target prediction and functional annotation. It uses the machine learning methods for the prediction of miRNA.	Nathan Wong and Xiaowei Wang(2015). Xiaowei Wang (2016)

3.4 Comparison and Validation of Target Genes

Target gene retrieved, against selected miRNA, from various databases were compared and validated. The venny tool was used to create the Venn diagrams against the ranked genes predicted from above databases and the database of autism spectrum disorder (ASD).

Target genes were retrieved against selected miRNA from TargetScan Human, miRDB and Diana. The genes were compared and validated. The venny tool was used to create the venn diagram to do the comparison against the ranked genes predicted from the already mentioned databases.

Table 3.3: Database and Tool for Comparisons of Candidate Genes Retrieved

Sr.no	**Database/ Tool**	**URL**	**Descriptions**	**References**
1	Autism Informatics Portal (AutDB)	http://autism.mind-spec.org/autdb/Welcome.do	It contains the annotated list of genes, involved in ASD.	Basu SN, Kollu R, Banerjee-BasuS.Nucleic Acids Res. 2009 Jan;37 (Database issue):D832-6.
2	Venny tool	http://bioinfogp.cnb.csic.es/tools/venny/	It is a tool used to create the venn diagrams, to obtain logical results among the collection of differentsets.	Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams.

3.5 Functional Annotation of Target Genes

The genes obtained from various databases were annotated through DAVID. It is clustering tool, which makes cluster of similar, redundant and heterogeneous genes based on functional annotation.

Table 3.4: Database for Functional Annotation of Candidate Target Genes

Sr.no	**Database**	**URL**	**Descriptions**	**References**
1	DAVID 6.8	https://david.ncifcrf.gov/tools.jsp	It is a database for Annotation, Visualization and Integrated Discovery. It has different sets of tools through which functional annotation is done to know about integrated pathways Behind the genes.	Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources.(2009)

3.6 Functional Annotation of miRNAs

The functional annotation of miRNAs was performed to identify and specify the putative functions of miRNA which was further divided as up regulated and down regulated miRNAs.

The comparisons with annotated genes was performed to predict the overlaps. Functional Annotation of miRNA gave different pathways against the miRNAs so that we could identify putative miRNA involved in certain pathway.

Table 3.5: Databases for Functional Annotation of miRNAs

Sr.no	Database	URL	Descriptions	References
1	Diana mir- Path v.3	http://snf-515788.vm.okeanos.grnet.gr/	DIANA-mirPath is a miRNA pathway analysis webserver. It predicts the miRNA targets which is already provided by the DIANA-microT-CDs algorithm. The interactions are combined which is result of merging and doing meta-analysis of these interactions.	Vlachos, IoannisS., KonstantinosZagganas, Maria D. Paraskevopoulou, GeorgiosGeorgakilas, DimitraKaragkouni, ThanasisVergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou(2015)
2	miRDB	http://www.mirdb.org/cgi-bin/search.cgi	miRDB is an online database for miRNA target prediction and functional annotation . It uses the machine learning methods for the prediction of miRNA. On this base it gives the target scores for each gene.	Nathan Wong and Xiaowei Wang(2015). Xiaowei Wang (2016)

3.7 Network and Pathway Enrichment

Pathways and network was used to predict functions of selected candidate gene using STRING.

Table 3.6: Database to Predict Pathways and Networks

Sr.no	Database	URL	Descriptions	References
1	STRING	https://string-db.org/	STRING (Search Tool for the Retrieval of Interacting Genes/Protein) is a database of known and predicted protein- protein interactions and explore GO with use of KEGG pathway. The associations involved in this are both physical and functional.	Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, von Mering C.(2017)
				Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta- Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M, Bork P, Jensen LJ, von Mering C.(2015)

Chapter 4

Results and Discussions

For the discovery of efficient diagnostic biomarker such as miRNAs, following analysis has been done to find out particular miRNAs which could be explored as potential diagnostic biomarker for ASD.

4.1 Identification of miRNA

Lists of 28, 28 and 41 miRNAs from miR2disease, phenomiR and HMDD respectively, were obtained. The three lists were combined and duplication was removed. Remaining 45 miRNAs were considered in final list. After that 12 miRNAs, failed to predict targets were further eliminated from the list, generating a resultant list of 33 miRNAs.

4.1.1 List of miRNAs Retrieved from miR2 Disease Database

The microRNAs (miRNAs) which were experimentally validated, involved in autism spectrum disorder (ASD) particularly present in blood, serum and plasma were selected from three different databases. The database involves, miR2Disease Base in which the miRNA were searched by disease name i.e. Autism Spectrum Disorder (ASD). The database showed the result by Pervasive Development Disorder

name and given the list of miRNAs related to ASD. The total 28 miRNAs was retrieved from the database related to ASD.

TABLE 4.1: List of miRNAs and their Expression Pattern Retrieved from miR2Disease

Sr.no	**miRNA ID**	**Detection Method for miRNA**	**Expression Pattern of miRNA**
1	hsa-miR- 15a	Microarray	Upregulated
2	hsa-miR 15b	Microarray	Upregulated
3	hsa-miR-21	Microarray	Upregulated
4	hsa-miR-212	Microarray	Upregulated
5	hsa-miR-431	Microarray	Upregulated
6	hsa-miR-432	Microarray	Downregulated
7	hsa-miR-484	Microarray	Upregulated
8	hsa-miR-539	Microarray	Downregulated
9	hsa-miR-550	Microarray	Downregulated
10	hsa-miR-598	Microarray	Upregulated
11	hsa-miR-652	Microarray	Downregulated
12	hsa-miR-7	Microarray	Upregulated
13	hsa-miR-93	Microarray	Downregulated
14	hsa-miR-95	Microarray	Upregulated
15	has-miR-106b	Microarray	Downregulated
16	hsa-miR-128a	Microarray	Upregulated
17	hsa-miR-129	Microarray	Upregulated
18	hsa-miR-181d	Microarray	Downregulated
19	hsa-miR-132	Microarray	Upregulated
20	hsa-miR-140	Microarray	Downregulated
21	hsa-miR-23a	Microarray	Upregulated
22	hsa-miR-27a	Microarray	Upregulated
23	hsa-miR-320a	Microarray	Downregulated
24	hsa-miR-148b	microarray	Upregulated
25	hsa-miR-106a	microarray	Downregulated
26	hsa-miR-146b	microarray	Downregulated
27	hsa-miR-193b	Microarray	Downregulated
28	hsa-miR-381	microarray	Downregulated

4.1.2 List of miRNAs Retrieved from PhenomiR Database

The miRNAs had been selected by selecting the disease list from database. After the selection of ASD in the query box a list was retrieved containing 28 miRNAs as shown in table 4.2.

TABLE 4.2: List of miRNAs and their Expression Pattern Retrieved from PhenomiR

Sr.no	miRNA ID	Detection Method for miRNA	Expression Pattern of miRNA
1	has-mir-106b	quantitative PCR	Downregulated
2	hsa-mir-106a	quantitative PCR	Downregulated
3	hsa-mir-128-1	quantitative PCR	Upregulated
4	hsa-mir-129-1	quantitative PCR	Upregulated
5	hsa-mir-132	quantitative PCR	Upregulated
6	hsa-mir-140	quantitative PCR	Downregulated
7	hsa-mir-146b	quantitative PCR	Downregulated
8	hsa-mir-148b	quantitative PCR	Upregulated
9	hsa-mir-15a	quantitative PCR	Upregulated
10	hsa-mir-15b	quantitative PCR	Upregulated
11	hsa-mir-181d	quantitative PCR	Downregulated
12	hsa-mir-193b	quantitative PCR	Downregulated
13	hsa-mir-21	quantitative PCR	Upregulated
14	hsa-mir-212	quantitative PCR	Upregulated
15	hsa-mir-23a	quantitative PCR	Upregulated
16	hsa-mir-27a	quantitative PCR	Upregulated
17	hsa-mir-320a	quantitative PCR	Downregulated
18	hsa-mir-381	quantitative PCR	Downregulated
19	hsa-mir-431	quantitative PCR	Upregulated
20	hsa-mir-432	quantitative PCR	Downregulated
21	hsa-mir-484	quantitative PCR	Upregulated
22	hsa-mir-539	quantitative PCR	Downregulated
23	hsa-mir-550-1	quantitative PCR	Downregulated
24	hsa-mir-598	quantitative PCR	Upregulated
25	hsa-mir-652	quantitative PCR	Downregulated
26	hsa-mir-7-1	quantitative PCR	Upregulated
27	hsa-mir-93	quantitative PCR	Downregulated
28	hsa-mir-95	quantitative PCR	Upregulated

4.1.3 List of miRNAs Retrieved from HMDD Database

The HMDD database provided a list by browsing the disease and searching by name as Autistic Disorder.

The list of miRNAs was obtained and downloaded.The Human microRNA Disease Database is a list of human microRNA (miRNA) and disease interactions that have been experimentally validated.

TABLE 4.3: List of miRNAs Retrieved from HMDD Database

Sr.no	**miRNA ID**	**Detection Method for miRNA**	**Expression Pattern of miRNA**
1	hsa-mir-106a	quantitative PCR	downregulated
2	hsa-mir-106b	quantitative PCR	downregulated
3	hsa-mir-129-1	quantitative PCR	downregulated
4	hsa-mir-129-2	quantitative PCR	downregulated
5	hsa-mir-132	quantitative PCR	downregulated
6	hsa-mir-132	quantitative PCR	upregulated
7	hsa-mir-140	quantitative PCR	downregulated
8	hsa-mir-146a	quantitative PCR	upregulated
9	hsa-mir-146b	quantitative PCR	downregulated
10	hsa-mir-146b	quantitative PCR	upregulated
11	hsa-mir-148b	quantitative PCR	downregulated
12	hsa-mir-15a	quantitative PCR	downregulated
13	hsa-mir-15b	quantitative PCR	downregulated
14	hsa-mir-181b-1	quantitative PCR	downregulated
15	hsa-mir-181d	quantitative PCR	downregulated
16	hsa-mir-193b	quantitative PCR	downregulated
17	hsa-mir-21	quantitative PCR	upregulated
18	hsa-mir-212	quantitative PCR	upregulated
19	hsa-mir-23a	quantitative PCR	downregulated
20	hsa-mir-23a	quantitative PCR	upregulated
21	hsa-mir-23b	quantitative PCR	upregulated
22	hsa-mir-27a	quantitative PCR	downregulated
23	hsa-mir-363	quantitative PCR	downregulated
24	hsa-mir-381	quantitative PCR	downregulated
25	hsa-mir-431	quantitative PCR	downregulated
26	hsa-mir-432	quantitative PCR	downregulated
27	hsa-mir-484	quantitative PCR	downregulated
28	hsa-mir-486	quantitative PCR	downregulated
29	hsa-mir-539	quantitative PCR	downregulated
30	hsa-mir-550a-1	quantitative PCR	downregulated
31	hsa-mir-550a-2	quantitative PCR	downregulated
32	hsa-mir-598	quantitative PCR	upregulated
33	hsa-mir-652	quantitative PCR	downregulated
34	hsa-mir-663a	quantitative PCR	upregulated
35	hsa-mir-7-1	quantitative PCR	upregulated
36	hsa-mir-7-2	quantitative PCR	downregulated
37	hsa-mir-7-3	quantitative PCR	downregulated
38	hsa-mir-92a-1	quantitative PCR	downregulated
39	hsa-mir-92a-2	quantitative PCR	downregulated
40	hsa-mir-93	quantitative PCR	downregulated
41	hsa-mir-95	quantitative PCR	downregulated

4.1.4 Elimination of Duplicated miRNA

The three different lists of miRNAs retrieved from three different databases were combined and redundancy was removed in which the miRNAs which were matched from all of three were kept in a single list. The origin and evolution of microRNA (miRNA) genes, which play important roles in tuning and buffering gene expression in a variety of essential cellular processes, has long attracted the attentions of evolutionary biologists. The duplicated found were 52 and were removed from total 97 miRNAs. Furthermore, the miRNAs are prioritized. After removal of 52 duplicates total 45 miRNAs were selected for further analysis.

Table 4.4: List of Resultant miRNA after Elimination of Duplicated miRNAs

Sr.no	**miRNA ID**	**Detection method for miRNA**	**Expression pattern of miRNA**
1	hsa-miR- 15a	microarray	upregulated
2	hsa-miR 15b	microarray	upregulated
3	hsa-miR-21	microarray	upregulated
4	hsa-miR-212	microarray	upregulated
5	hsa-miR-431	microarray	upregulated
6	hsa-miR-432	microarray	downregulated
7	hsa-miR-484	microarray	upregulated
8	hsa-miR-539	microarray	downregulated
9	hsa-miR-550	microarray	downregulated
10	hsa-miR-598	microarray	upregulated
11	hsa-miR-652	microarray	Downregulated
13	hsa-miR-93	microarray	downregulated
14	hsa-miR-95	microarray	upregulated
15	has-miR-106b	microarray	downregulated
16	hsa-miR-128a	microarray	upregulated
17	hsa-miR-129	microarray	upregulated
18	hsa-miR-181d	microarray	downregulated
19	hsa-miR-23a	microarray	upregulated
20	hsa-miR-27a	microarray	upregulated
21	hsa-miR-320a	microarray	downregulated
22	hsa-miR-148b	microarray	upregulated
23	hsa-miR-106a	microarray	downregulated
24	hsa-miR-146b	microarray	downregulated

Continued Table 4.4: List of Resultant miRNA after Elimination of Duplicated miRNAs

Sr.no	**miRNA ID**	**Detection method for miRNA**	**Expression pattern of miRNA**
25	hsa-miR-193b	microarray	downregulated
26	hsa-miR-381	microarray	downregulated
27	hsa-mir-128-1	quantitative PCR	Upregulated
28	hsa-mir-129-1	quantitative PCR	Upregulated
29	hsa-mir-551-b	quantitative PCR	downregulated
30	hsa-mir-7-1	quantitative PCR	Upregulated
31	hsa-mir-129-2	quantitative PCR	downregulated
32	hsa-mir-146a	quantitative PCR	upregulated
33	hsa-mir-181b-1	quantitative PCR	Downregulated
34	hsa-mir-23b	quantitative PCR	Upregulated
35	hsa-mir-363	quantitative PCR	Downregulated
36	hsa-mir-486	quantitative PCR	downregulated
37	hsa-mir-550a-1	quantitative PCR	downregulated
38	hsa-mir-550a-2	quantitative PCR	downregulated
39	hsa-mir-663a	quantitative PCR	upregulated
40	hsa-mir-7-2	quantitative PCR	downregulated
41	hsa-mir-7-3	quantitative PCR	downregulated
42	hsa-mir-92a-1	quantitative PCR	downregulated
43	hsa-mir-92a-2	quantitative PCR	downregulated
44	hsa-miR-132	microarray	upregulated
45	hsa-miR-140	microarray	downregulated

4.2 Prioritization of Selected miRNAs

A short listed miRNAs after redundancy removal were prioritized based on target gene list and statistical significance of target gene. miRNAs were eliminated on following criteria.

- After target prediction.
- If the target prediction software did not generate a statistically significant gene list using the cut-off criteria.

TABLE 4.5: Summary of miRNA prioritization

Procedure	**Number of miRNAs eliminated**	**Total miRNAs**
miRNAs collected from Databases	0	97
Elimination of duplicates	52	45
Target Predictions.	12	33

4.3 Target Gene Prediction

Target prediction had been performed for the selected miRNAs, indicating that which miRNA is involved with genes in these databases. After integration of three target gene software programs, total 25,454 genes were attained. Moreover, duplicated genes were eliminated and the remaining genes were only kept if all of them showed their presence in three predicted algorithms, giving putative list of target genes. The prediction of target was done separately for both upregulated and downregulated miRNAs. The known genes for comparison with candidate genes were obtained from Autism Informatics portal (AutDB).

The selected targeted genes was then compared against 991 known genes associated with ASD, which will be acquired from the Autism Database (AutDB) to look over either any of predicted target genes were related to ASD or not. About 468 and 200 genes were found to overlap with autistic known genes. Every database has different criteria for prediction of genes. MiRNA target sites were predicted through three miRNA target prediction softwares, easily available online.

Total three gene lists were attained for each miRNA. Separate gene lists of upregulated and downregulated miRNAs were obtained. However, authentic and reliable target genes were selected according to following criteria:

1. Only those genes were selected from each prediction program if they come under the cut off criteria.

2. Genes were selected only if all the three programs predicted them.

3. Thereafter, the genes obtained were combined resulting in total of 25454 genes and analyzed for duplicates. 15780 duplicates were found and removed in MS Excel 2010 using the elimination shortcut "**Alt+A+M**", the unique 9676 genes were obtained and sorted having 3371 down regulatory and 6302 upregulated miRNA target genes.

TABLE 4.6: Target Genes Obtained from Three Predictiondatabases, Before Removal of Duplications

Databases	**Genes Count**
Target Scan	10,000
mirdb	4,454
Diana Micro-T	11,000

4.4 Comparison of Selected Genes with ASD Reported Genes

The prioritized genes were compared with 991 known genes associated with ASD, attained from the Autism Database (AutDB) to look over either the predicted target genes were associated to ASD previously or not. The VENNY tool v. 2.1 was used to create Venn diagrams shown in **Figure 4.1**.

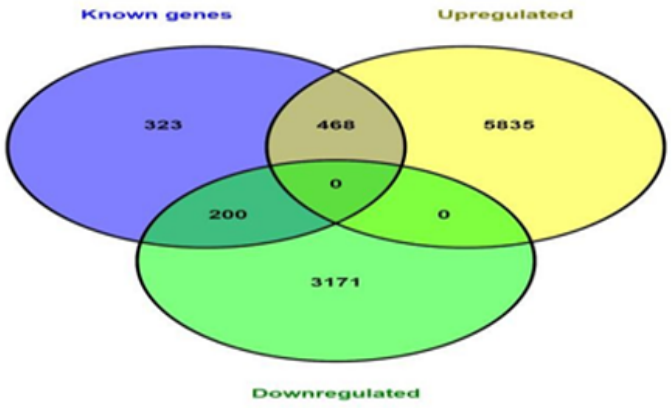

FIGURE 4.1: Comparison of exclusively known genes associated with ASD (blue) to the predicted down (green) and up- regulated (yellow) gene lists before functional annotation. There are 200 genes, and 468 genes overlapping from down- and up-regulated, respectively

4.5 Functional Annotation of Predicted Genes and miRNAs

4.5.1 Target Genes

Functional Annotation was carried for the entire gene list separately by using clustering tool available on DAVID (Version 6.8) (The Database for Annotation, Visualization and Integrated Discovery).

The clustering tool grouped genes that may be linked biologically i.e. similar, redundant and heterogeneous genes will be grouped in the same cluster.

For functional annotation parameters were remain Default. "Medium" classification parameter and raw p values were used. Moreover, gene clusters with enrichment score >1.3 [117], for biological actions ("GO-TERM- BP") and molecular function ("GO-TERM-MF") were selected.

In this way gene list was prioritized further. The prioritize gene list was inserted in to VENNY tool and compared to the 991 susceptible known genes list. The genes which show overlapping were specially observed as significant and used for further study.

After finding clusters duplicates were removed and separation of gene symbolic in EXCEL sheet was done by using "=LEFT (A3, FIND("", A3)-1)" formula. Then the list of gene symbol id for both up and down-regulated genes were compared with 991 known genes in VENNY tool. 148 upregulated genes and 41 downregulated genes were overlapped with known genes.

148 upregulated and 41 downregulated genes were then used in STRING for further analysis for the determination of proper conclusion. The selected genes were then used to predict significant miRNA used as a biomarker.

Predicted miRNAs will be used for early diagnosis of Autism spectrum disorder. If proper validation of miRNAs has been occurred then it will be used for further products.

Annotation Cluster 8	Enrichment Score: 4.37			Count	P_Value	Benjamini
GOTERM_BP_FAT	neuron differentiation	RT		102	1.5E-6	3.0E-4
GOTERM_BP_FAT	cell projection organization	RT		87	5.0E-6	7.8E-4
GOTERM_BP_FAT	neuron development	RT		81	7.2E-6	1.1E-3
GOTERM_BP_FAT	neuron projection development	RT		65	8.5E-6	1.2E-3
GOTERM_BP_FAT	cell projection morphogenesis	RT		62	1.6E-5	1.9E-3
GOTERM_BP_FAT	cell morphogenesis	RT		82	2.6E-5	2.8E-3
GOTERM_BP_FAT	neuron projection morphogenesis	RT		55	2.8E-5	2.8E-3
GOTERM_BP_FAT	cell part morphogenesis	RT		63	3.3E-5	3.2E-3
GOTERM_BP_FAT	cell morphogenesis involved in neuron differentiation	RT		52	1.3E-4	1.0E-2
GOTERM_BP_FAT	cellular component morphogenesis	RT		86	1.7E-4	1.3E-2
GOTERM_BP_FAT	axonogenesis	RT		47	4.8E-4	2.8E-2
GOTERM_BP_FAT	cell morphogenesis involved in differentiation	RT		56	6.2E-4	3.2E-2
GOTERM_BP_FAT	axon guidance	RT		29	1.3E-3	5.5E-2

FIGURE 4.2: Description of gene cluster of biological process of upregulated targeted genes retrieved from DAVID

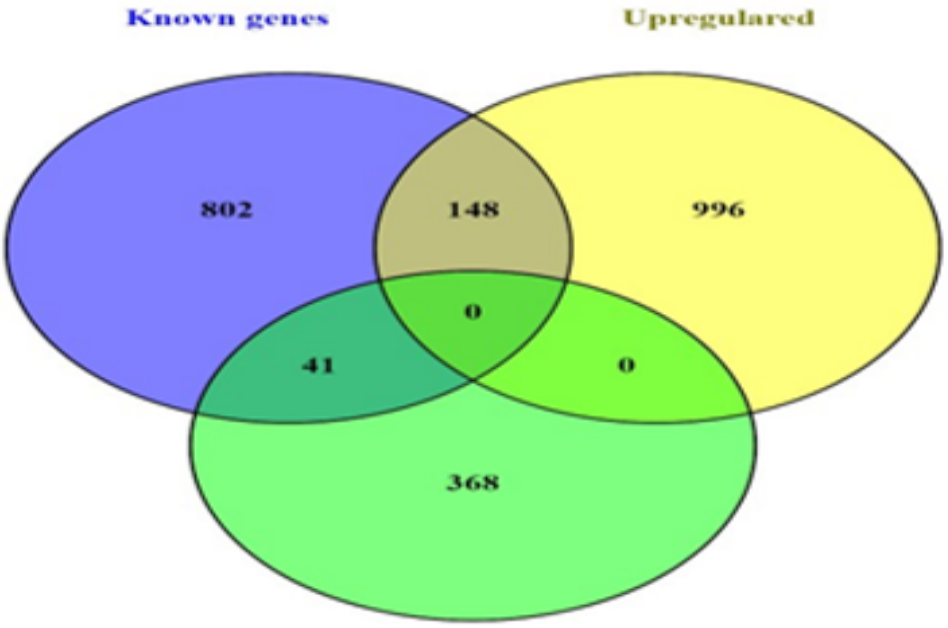

FIGURE 4.3: Comparison of ASD known susceptible genes (blue) to the predicted upregulated (yellow) and downregulated (green) gene list after the step of functional annotation.148 genes were observed to still overlap the known genes associated with Autism from upregulated and 41 from downregulated

Table 4.7: List of 41 Downregulated Genes Overlapped with Known Genes

ANKRD11	**OTX1**
APP	P2RX4
ARHGAP33	PCDH8
ARX	PLXNA3
BBS4	PLXNB1
CECR2	PRKDC
DLG1	SHOX
DLX6	SLC6A4
DNER	SMC1A
DPYD	TERF2
DRD1	THRA
DRD2	TNN
EGR2	TSC1
EPS8	TUBGCP5
ERMN	WNT1
ESR1	XIRP1
FLT1	YWHAE
HDAC4	YY1
HTR2A	NR2F1
ITPR1	NRP2
NEFL	

Table 4.8: List of 148 Upregulated Genes Overlapped with Known Genes

ACTN4	HUWE1	HERC2	SMARCA2
ADRB2	IFNG	HIVEP3	SOX5
AFF2	ITGA4	NRXN1	STX1A
AFF4	JARID2	NRXN3	TAF1
ANK3	JMJD1C	NSD1	TBL1X
ANXA1	KCNQ3	NTRK2	TBL1XR1
APC	KIF5C	PAFAH1B1	TBR1
AR	LMX1B	PAH	TCF7L2
ARID1B	LRP2	PAX5	THBS1
ASH1L	MAOA	PAX6	TNRC6B
ATRX	MAP2	PEX7	TOMM20
ATXN7	MAPK1	PLCB1	TSC2
AVPR1B	MBD1	POLA2	TYR
BCL11A	MBD3	PON1	WNK3

Continued Table: 4.9 List of 148 Upregulated Genes Overlapped with Known Genes

BCL2	MDGA2	POT1	XPO1
BDNF	MED13	POU3F2	ZNF462
BRAF	MFRP	PRICKLE2	DDX11
CACNA1A	MIB1	PRUNE2	DLGAP1
CACNA1B	MTF1	PSMD12	DLX2
CACNA1E	MTX2	PTEN	DMD
CADM1	MYT1L	PTGER3	DMXL2
CAMK2A	NAA15	PTGS2	GRIN2A
CAMK2B	NBEA	PTK7	GRIN2B
CAMK4	NELL1	PTPN11	GRM4
CD44	NF1	PTPRC	GSK3B
CDKN1B	NLGN1	RAC1	HCFC1
CEP290	NLGN3	RANBP17	SLC12A5
CHD1	NR1D1	RB1CC1	SLC1A2
CHD2	NRG1	RFX3	SLC6A1
CHD7	EIF4E	RIMS1	SLIT3
CHD8	EP300	ROBO1	SMAD4
CLASP1	EPC2	ROBO2	SHANK3
CREBBP	ERBB4	RORA	SIK1
CTCF	EXT1	RPS6KA3	SIN3A
CTNNB1	FGD1	SATB2	SIK1
CTTNBP2	FOXP1	SETD2	SIN3A
CUL3	GPX1	SHANK3	
CUX1	GRIA1		
DCX	GRIN1		

4.6 Functional Analysis of miRNAs

Then functions of up regulated and down regulated miRNAs were determine through Diana-mirPath. The possible functions for the 17 down-regulated miRNA and 16 up-regulated miRNAs were queried separately using default settings.

miRNA target prediction and functional enrichment analysis of miRNA targets are typically part of in silico functional analysis of miRNAs. To determine the regulatory impact of a specific miRNA, one typical approach is to compare miRNA and mRNA expression.

For significant and efficiency of the pathways, a heatmap was generated after the pathways union, where 13 and 14 pathways were shown, which are given in figure 4.4 and 4.5.

TABLE 4.9: Pathways Involved in Down Regulated miRNAs

Pathways Involved in Down Regulated miRNAs	**Pathways I in Up Regulated miRNAs**
Proteoglycans in cancer	Glycosphingolipid biosynthesis lacto and neolacto series.
Pathways in cancer	Hippo signaling pathway.
nicotine addiction	Proteoglycans in cancer.
TGF-beta signaling pathway	Lysine degradation.
Signaling pathways regulating pluripotency of stem cells.	Glioma.
Hippo signaling pathway	Signaling pathways regulating pluripotency of stem cells.
fatty acid metabolism	TGF-beta signaling pathway
Fatty acid biosynthesis	Morphine addiction.
Thyroid hormone signaling pathway	Amphetamine addiction.
Glioma	ECM- receptor interactions
Melanoma	fatty acid degradation
Lysine degradation	N-Glycan biosynthesis.
Axon guidance	Metabolism of xenobiotic by cytochrome p450
Adherens junction	

The study explored that miRNA functions and target genes functions are linked such as identified target genes are associated with nerve signaling and the pathways of miRNA discussed also have nerve signaling pathways involved in axon guidance and other linked pathways.

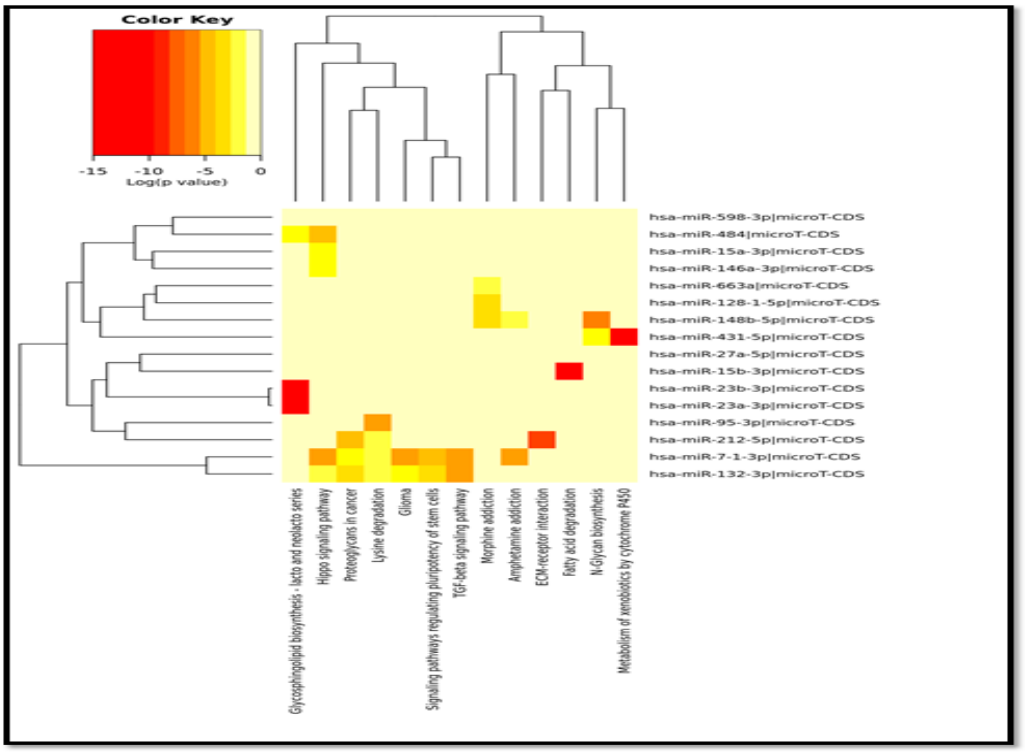

FIGURE 4.4: Functional annotations of upregulated miRNAs and their involvement in different pathways, using Diana mirPath showing the heat map

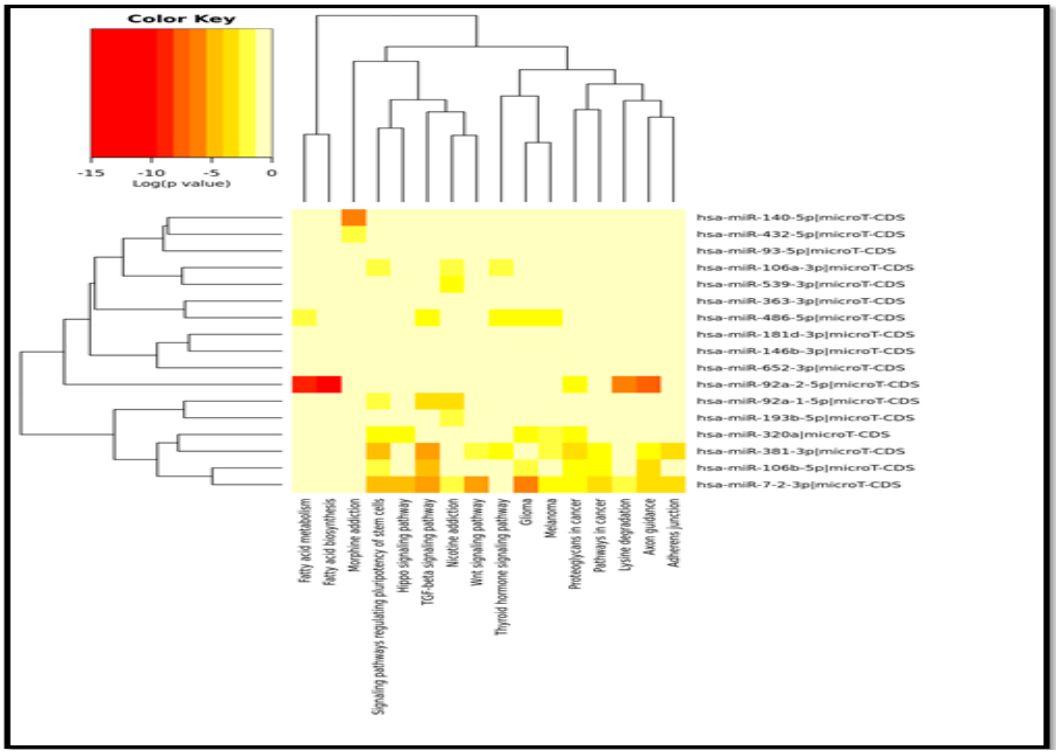

FIGURE 4.5: Functional annotations of downregulated miRNAs and their involvement in different pathways, using Diana mirPath showing the heat map

4.7 Network and Enrichment Analysis

The STRING version 11 (Search Database for the Retrieval of Interacting Genes) is a web based tool which is greatly used for analyzing protein interactions (PPI), gene ontology (GO) annotations, and KEGG pathway analysis. Hence, KEGG pathway analysis was done separately for downregulated and upregulated lists of target genes. STRING database anticipate both investigational and predicted associations and interactions by collecting information from certain techniques i.e. co-expression analysis, high throughput techniques, genomics and published literature. Up regulated and down regulated genes were then separately queried and mapped to STRING for gene network enrichment and KEGG pathway. The genes were copied from Excel sheet and pasted into the search box, in the "multiple names" tab in STRING. Pathways with $p<0.05$ were analyzed as playing significant role. Interaction analysis was done under default settings and confidence score set to >0.4. Based on the results the downregulated geness were predicted to be associated in the Gap junction, Calcium signaling pathway, Neuroactive ligand-receptor interaction and Serotonergic synapse.

Significant pathways for upregulated gene list were Long term potentiation, Went signaling pathway, Prostrate cancer, Neurotrophin signaling pathway , Calcium signaling pathway, Glutamatergic synapse, Dopaminergic synapse, Melanogenesis, Amyotrophic lateral sclerosis, Colorectal cancer, Amphetamine addiction, Adherence junction, HIF-1 signaling pathway, Cholinergic synapse, Pathways in cancer, ErbB signaling pathway, Thyroid hormone signaling pathway, Circadian entrainment, Nicotine addiction, Alcoholism, MicroRNAs in cancer, Endometrial cancer, Focal adhesion, mTOR signaling pathway, Proteoglycans in cancer, Renal cell carcinoma, Epstein-Barr virus infection, MAPK signaling pathway, Serotonergic synapse, Rap1 signaling pathway, TGF-beta signaling pathway, FoxO signaling pathway, Cocain addiction, Thyroid cancer, Huntington disease, Long term depression, Retrograde endocannabinoid signaling, Hepatitis B, Glioma, Chronic myeloid leukemia, P13 K- Akt signaling pathway, Arrhythmogenic right ventricular cardio myopathy, Tuberculosis, Axon guidance, Type II diabetes mellitus, Adrenergic

signaling in cardiomyocytes, Neuroactive ligand-receptor interaction, Oxytocin signaling pathway, Oocyte meiosis, Pancreatic cancer, Ras signaling pathway, Cell cycle, Alzheimers disease, Influenza A, Leukocyte transendothelial migration, Leishmeniasis, Viral carcinogenesis, Natural killer cell mediated cytotoxicity, Bladder cancer, Insulin signaling pathway, Cell adhesion molecules, GABAergic synapse, Small cell lung cancer, GnRH signaling pathway, Regulation of actin cytoskeleton, Chemokine signaling pathway, Systemic lupus erythematosus, Lysine degradation, VEGF signaling pathway Taste transduction, Phenylalanie metabolism, Basal cell carcinoma, Acute myeloid leukemia, Shigellosis, Herpes simplex infection, HTLV-1 infection, p53 signaling pathway, Osteoclast differentiation, Tight junction, Vascular smooth muscle contraction, B cell receptor signaling pathway, Melanoma, Thyroid hormone synthesis and Gastric acid secretion. Moreover STRING analysis revealed 13 interactions observed for downregulated and 231 for upregulated miRNA target genes as shown in figure 4.6 and 4.7 respectively. Interestingly, the two genes shown for ASD susceptibility (HTR2A, SLC6A4) were shown to interact with each other.

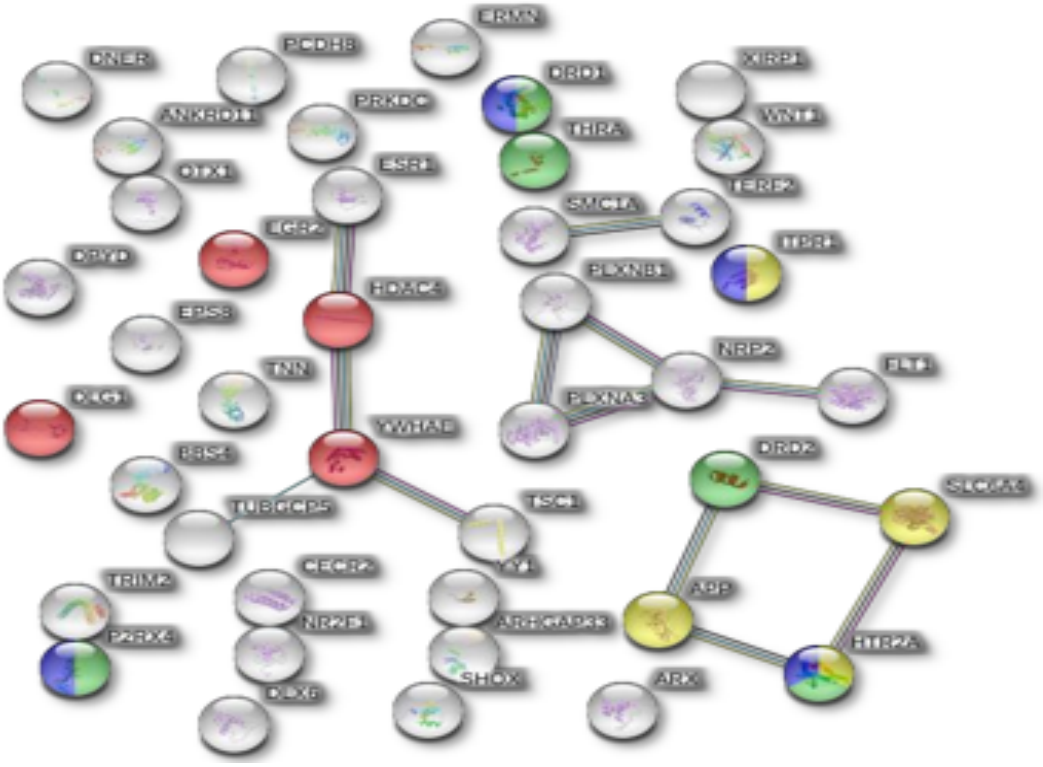

FIGURE 4.6: STRING analysis of interactions in the downregulated miRNA target genes. 13 interactions were observed for downregulated genes

4.7.1 HTR2A (5-Hydroxytryptamine (Serotonin) Receptor2a)

HTR2A gene is involved in most of the downregulated miRNA targeted genes pathways. Serotonin (5-hydroxytryptamine, 5HT) is a conventional monoamine involved broadly in different physiological processes which includes the social behaviors, cardiovascular regulation, homeostasis, gastrointestinal functions, circadian rhythms, cognition and mood swings. Serotonin signaling is also responsible and implicates its participation in the regulation of many neurodevelopmental processes. Some evidences also propose that it might play an important role in the developmental programming of childhood as well as adult-onset mental conditions [106]. Previous studies claim that seven candidate genes involved in neurotransmission of brain, serotonin pathways and mapping to autism linkage (SLC6A4, HTR1A, HTR1D, HTR2A, HTR5A, TPH1 and ITGB3) are responsible for etiology of autism [107]. Increased levels of platelets serotonin (5-HT) have been found in ASD patients. Specifically 5-HT transporter gene (SLC6A4) variants, initiate a transporter to reuptake the putative functions [108].

The serotonin transporter gene (SLC6A4) is a candidate gene in autistic disorder patients which are based upon neurochemical and neuroendocrine studies, and also the effectiveness of potential serotonin transporter inhibitors in minimizing ritualistic aggression behavior [109]. In ASD patients disturbed brain and peripheral serotonin homeostasis had been found. Serotonin receptor 2A (HTR2A) is responsible for regulation of central and peripheral serotonin homeostasis. It also modifies expression in autistic subjects. It has been seen and claimed that HTR2A gene act as a major candidate gene for the serotonin disturbance in autistic patients. Moreover, several studies yielding so far uncertain outcomes, have endeavored to associate autism with a functional SNP - 1438 G/A (rs6311) in the HTR2A promoter region [110][111].

MAPK1 and PLCB1 genes were highly pathway enrichment genes. Out of total 85 pathways these above mentioned genes show involvement in 30-40 pathways respectively.

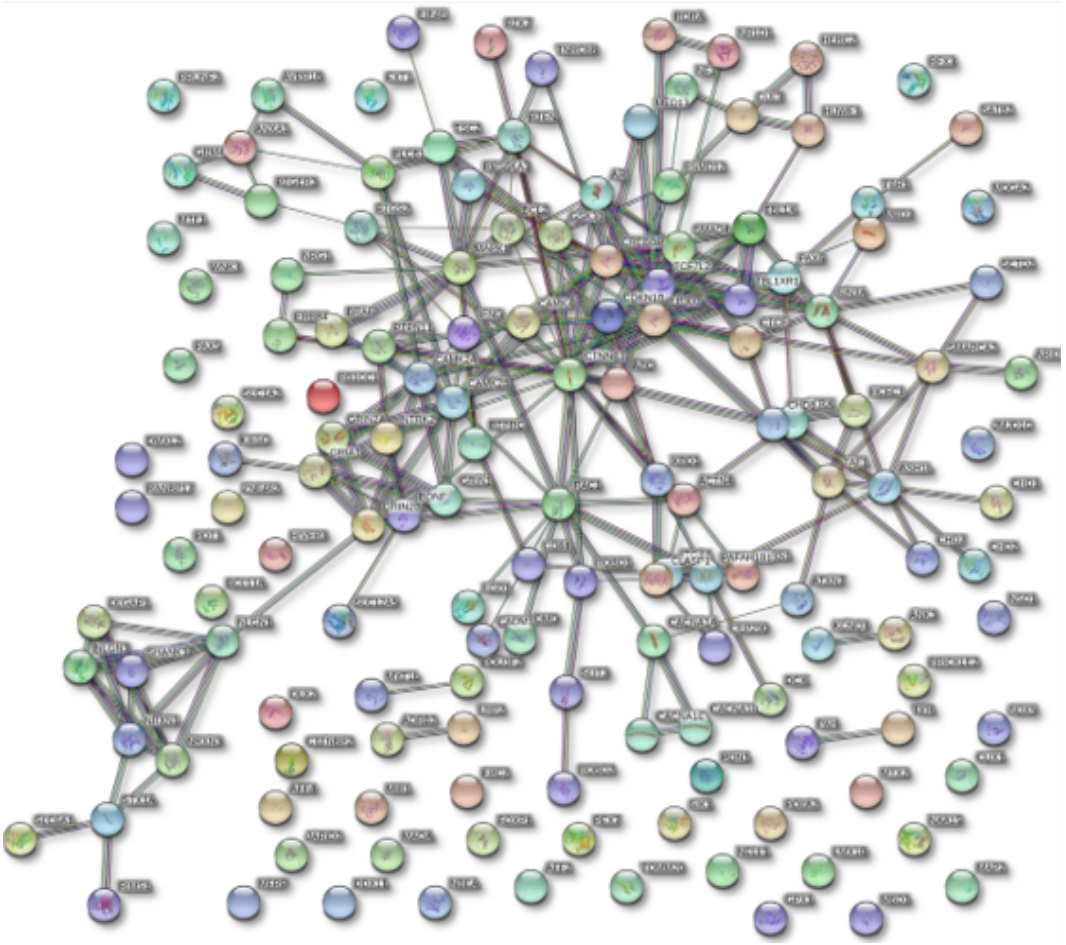

FIGURE 4.7: STRING analysis of pathway enrichment and interaction in the upregulated miRNA target genes. 231 interactions were observed for upregulated genes

4.7.2 MAPK1 (Mitogen-Activated Protein Kinase1)

Mitogen-activated protein kinase 1, serine/threonine kinase which acts as a key component of the MAP kinase signal transduction pathway. MAPK1/ERK2 and MAPK3/ERK1 are the 2 MAPKs plays the major role in the MAPK/ERK cascade. They are also part of signaling cascade which are initiated by activated KIT and KITLG/SCF. The MAPK/ERK cascade arbitrates various biological functions which includes the survival cell growth, adhesion, and differentiation by the regulation of transcription, translation, cytoskeletal rearrangements depending on the cellular context of the cell. However previous studies and its analysis suggests that MAPK signaling and Calcium Signaling pathways strongly associated with ASD [112].

4.7.3 PLCB1 (Phospholipase C, beta 1(Phosphoinositide-Specific)

The second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) production is arbitrated by activated phosphatidylinositol-specific phospholipase C enzymes. Some, previous studies claim that rare copy number variants have been highly responsible for ASD. These variants affect great number of genes including PLCB1. PLCBI is enriched in causing gene disruptive events in ASD [113]. Chromosomal location of PCLB1 is 20p12.3.

4.8 miRNA Identification and Target Prediction

The genes attained from Diana micro-T, Target scan Human and miRDB was shortened from 25,454 genes to 41 downregulated and 148 unregulated genes against down regulated and upregulated miRNAs.

The downregulated miRNA target gene list had 1 known gene HTR2A found to play a significant role in ASD. Whereas the upregulated miRNA target gene list had 2 known genes: MAPK1 and PLCB1. Using a methodology a number of 4 miRNAs was identified i.e. *miR-7, mir-23, miR-106 and miR-93.*

The miRNA of particular interest were miR-106b-5p and miR-93-5p as only these 2 miRNAs related with predicted target genes. Genes are involved in possible molecular activities and biological actions that might be involved in the occurrence of ASD. The ability of miRNAs

to regulate broad molecular pathways in response to environmental stimuli makes them an intriguing player in ASD, a disorder characterized by genetic predisposition with ill-defined environmental triggers.

In addition, the availability and extracellular stability of miRNAs make them an ideal candidate for biomarker discovery. 3 or more studies identified miR-106b as significant biomarker for ASD [114][28].

TABLE 4.10: Novel Biomarkers (miRNAs) and their Predicted Targeted Genes

miRNA Used as Biomarker	**Predicted Targeted Genes**
miR-106b-5p, miR-93-5p	HTR2A
	MAPK1
	PLCB1

Hence, it is concluded that miR-106b-5p and miR-93-5p can be used as novel biomarker in ASD due to their intimacy in all of 3 predicted genes. Several previous studies identified these miRNAs as playing significant role in Autism [115]. As it is discussed in previous section that there is no blood test for ASD due to which early treatment of ASD is not possible. The analysis identified the efficient diagnostic biomarkers (miR-106b-5p,miR-93-5p) associated with ASD which can be used for earlier treatment as miRNAs play an important role in central nervous system, development and function.

We concluded that experiment analysis of three genes HTR2A, MAPK1 and PLCB1 could be determined by miR-106b-5p and miR-93-5p.These biomarkers could be detected in blood.

Chapter 5

Conclusion and Future Work

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder which includes deficits in social interactions, language impairments e.g. verbal and nonverbal communications. Person suffering from ASD is unable to maintain, understand or develop relationships. Both genetic and environmental factors are involved in onset of ASD. Several studies reported that fluctuations in gut microbiota also cause ASD. Behavioral based treatments are so far developed for the treatment and diagnosis of ASD. Psychiatrist mostly use questionnaires for the detection of disease, and it's possible after the age of 02 years or when child becomes able to speak. Otherwise at early stages diagnosis of ASD in individual is not an easy job. So far there is no blood test for the diagnosis of ASD at prenatal stage or early stages. For this purpose scientific community is trying harder to find out certain types of biomarker for the early diagnosis of ASD. Biomarker is any type of biological molecule e.g. gene, mRNA, miRNA and different body fluids which fluctuates in the body from the normal state in the presence of specific disease. Biomarkers are very helpful for clinical purposes as they can predict developmental changes and biomarkers can easily detect disease at early stages. Biomarkers are of different types e.g. diagnostic biomarkers, prognostic biomarkers and theranostic biomarker. So, there is a dire need of biomarker for the efficient and early diagnosis of ASD and to prevent the disorder. For this we have used insilico approach to identify biomarker as we have focused on miRNA. miRNAs are small non coding regions of RNA which are approximately made up of (18

-22) nucleotides, and involved in the regulation of more than half of the genes in human cell. They suppress translation and initiate degradation of mRNAs when complementary binding occurs. This deregulation of the expression of miRNA causes disease. By using insilico techniques firstly we selected miRNAs which are involved in ASD. Then we predict target genes based on selected miRNAs. Then we did functional analysis of target genes and miRNA. In this way we were able to identify biomarker for ASD.

The first objective was to select the miRNAs involved in ASD. To achieve this objective three different miRNAs detection tools were used e.g. miR2disease, phenomiR and HMDD. The three lists from the tools were combined and duplication was removed. The miRNAs which were experimentally validated, present in blood, serum and plasma of ASD patient were selected.

The second objective was to predict target genes based on selected miRNAs. After integration of three target gene software programs, total 25,454 genes were attained. Moreover, duplicates were removed and the remaining genes were only kept if all of them showed their presence in three predicted algorithms, giving putative list of target genes. . The prediction of target was done separately for both upregulated and downregulated miRNAs. Then comparison of selected genes with ASD reported genes from AutDB.

The third objective was functional annotation. Clustering tool DAVID was used for the annotation of genes and miRNA functions were determined by Diana-mirPath. Then network and enrichment analysis has been done by using STRING .Interactions for both down-regulated and up-regulated miRNAs were observed by networks.01 known gene HTR2A for downregulated and 02 knowm genes MAPK1 and PLCB1 for upregulated were identified .The miRNA of particular interest were miR-106b-5p and miR-93-5p as only these 2 miRNAs link the predicted target genes.

For the future work experimental validation of these biomarkers is suggested to validate their role in efficient diagnosis of ASD. The laboratory experiments can ultimately result in commercial products in future.

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