CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Analysis of Gut Microbiome Interactions in Autism Spectrum Disorder (ASD)

by

Ayesha Aftab

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2020

Copyright © 2020 by Ayesha Aftab

All rights reserved. No part of this thesis may be reproduced, distributed, or transmitted in any form or by any means, including photocopying, recording, or other electronic or mechanical methods, by any information storage and retrieval system without the prior written permission of the author.

I would like to dedicate this to my parents and teachers

CERTIFICATE OF APPROVAL

Analysis of Gut Microbiome Interactions in Autism Spectrum Disorder (ASD)

by

Ayesha Aftab

(MBS181012)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Saadia Andleeb	NUST, Islamabad
(b)	Internal Examiner	Dr. Arshia Amin Butt	CUST, Islamabad
(c)	Supervisor	Dr. Syeda Marriam Bakhtiar	CUST, Islamabad

Dr. Syeda Marriam Bakhtiar
Thesis Supervisor
June, 2020

Dr. Sahar Fazal
Head
Dept. of Biosciences & Bioinformatics
June, 2020

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health & Life Sciences
June, 2020

Author's Declaration

I, **Ayesha Aftab** hereby state that my MS thesis titled "**Analysis of Gut Microbiome Interactions in Autism Spectrum Disorder (ASD)**" is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/abroad.

At any time if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my MS Degree.

(Ayesha Aftab)

Registration No: MBS181012

Plagiarism Undertaking

I solemnly declare that research work presented in this thesis titled "**Analysis of Gut Microbiome Interactions in Autism Spectrum Disorder (ASD)**" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been dully acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS Degree, the University reserves the right to withdraw/revoke my MS degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized work.

(Ayesha Aftab)

Registration No: MBS181012

Acknowledgements

I would like to give all the praise to **Allah**, the most merciful and beneficent for blessing me the ability to complete this work and all the respect to His Holy Prophet who enabled us to recognize our creator.

I would like to acknowledge my supervisor and mentor, **Dr. Syeda Marriam Bakhtiar**, who helped me a lot in completion of this thesis as this would not be possible without her continuous guidance and moral support. I would also like to acknowledge all the faculty members of Capital University of Science and Technology who provided me with guidance throughout my MS.

A word of thanks goes to all my friends especially Nosheen, Maham, Shabana and Maria for their moral support.

I gratefully acknowledge and thank my siblings for their praiseworthy contribution, love and moral support. I have no words to express my gratitude for my parents, their love, care, support, encouragement and prayers have always enlightened my way throughout my life.

(Ayesha Aftab)
Registration No: MBS181012

Abstract

Autism spectrum disorders, is defined as a group of neurological disorders related to social behavior, issues in communication and cognitive abilities. The world-wide prevalence and financial burdens of this disorder are increasing day by day. But there is no proper treatment available for this disorder because the theories about etiology of ASD are changing throughout many past years. ASD is thought to be resultant of multiple factors like genetic, epigenetics and environmental factors. Due to this multifactorial pathophysiology of Autism, it is important to understand the role of these factors in development of autism. The environmental factors have been reported to play important roles in increasing the risk of ASD, these factors comprises of vaccination, exposure to pesticides, food habits, infections, use of antibiotics. This is also worthy to note that gut microbiota June play a significant role in aggravating the risk for developing ASD but the underlying mechanism of its involvement is not clear yet. This study is designed to find out the mechanisms by which gut microbiota is involved in causing ASD. It utilized metabolites that are products of gut microbiota produced as a result of food fermentation, for finding out the etiological basis of ASD. To attain this goal, an in silico methodology has been designed by exploiting physico-chemical, genetic and functional data of gut microbial metabolites in the form of protein–protein interactions, metabolic pathways and annotations. For this purpose, gut microbial metabolites were retrieved and analyzed for having some connections at both genetic and functional level. This study found 13 metabolites predicted to be associated with ASD because of having genetic connections while 7 metabolites have shown functional connections with ASD. The contribution of this study is the identification of the microbial metabolites present in gut that are playing role in ASD. This study provides the exploration of ASD etiology that will enable the researchers to find out the possible targeted treatments for this disorder.

Contents

List of Figures

List of Tables

Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
BL	Basolateral
CM	Centromedial
EEG	Electroencephalography
GI	Gastrointestinal
GO	Gene Ontology
MRI	Magnetic Resonance Imaging
PDD	Pervasive Developmental Disorders
PFC	Prefrontal Cortex

Chapter 1

Introduction

Cognition is a set of activities that are performed by human brain in order to carry out daily tasks such as to think, to plan, to remember things, idea generating, having creativity, giving attention. Problems related to cognition results into difficulty in performing everyday activities even though no stress is present. Among the highly occurring disorders involving cognitive issues, Autism spectrum disorders is an an important one [1].

1.1 Autism Spectrum Disorder

Autism spectrum disorder is defined as group of many neurological disorders related to social behavior, issues related to cognition and communication. The individuals affected by this disorder face problems in showing interaction with people. They also have interest in a small set of activities and they present a specific behavior for most of the times. The characteristics of the disease can be clearly observed in infants to three years old children. The symptoms of ASD are lack of eye contact with others, inability to control their emotions, face problems in understanding other people 's emotions and show interest a narrow set of activities [2],[3] the symptoms of ASD in affected individuals of different age groups is given in figure 1.1.

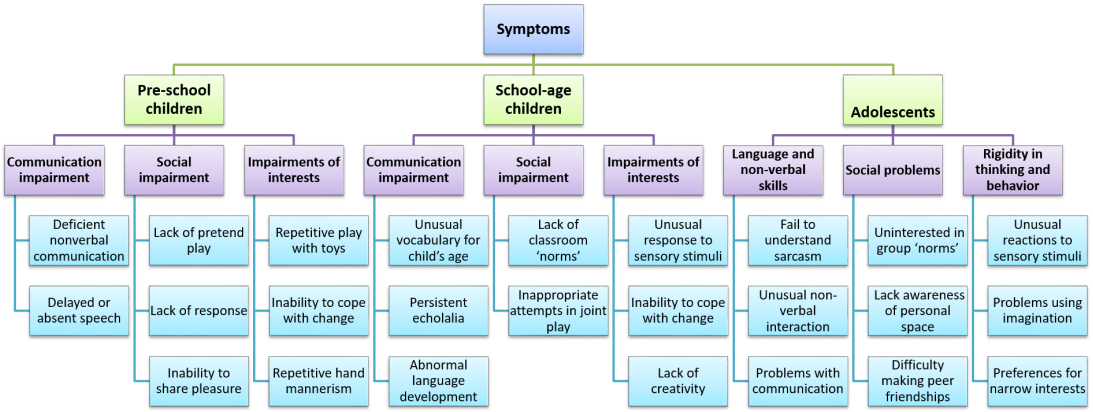

FIGURE 1.1: Symptoms of ASD in different age groups

1.1.1 Prevalence of ASD

The prevalence of autism is different across the world, but according to an estimate it is present in 1 to 2 percent population in overall the world [4].From last twenty years, there has been observed an increase in rate of autism. The National Health Center for Health Statistics measured the autism rate in 2016 and found that it occurs one child among every thirty six children [5]. World-wide prevalence of Autism spectrum disorder is shown in figure 1.2.

1.1.2 Effects of ASD Observed at Family Level and Society Level

The autistic child not only faces issues in his/her life but becomes a burden on family members also, because the living routine of family members is also disturbed by the autistic child in many ways. The problems faced by the autistic child's family are the special attention they need to provide their child in order to give support to him/her so that he or she can at least perform the basic activities of normal life. This special attention not only requires a lot of time but also require extra money to support their child such as treatment costs, care centers etc. The persons suffering from autism also act as a financial burden on society, as it has been recorded that United States spends 250 billion dollars on ASD children yearly.

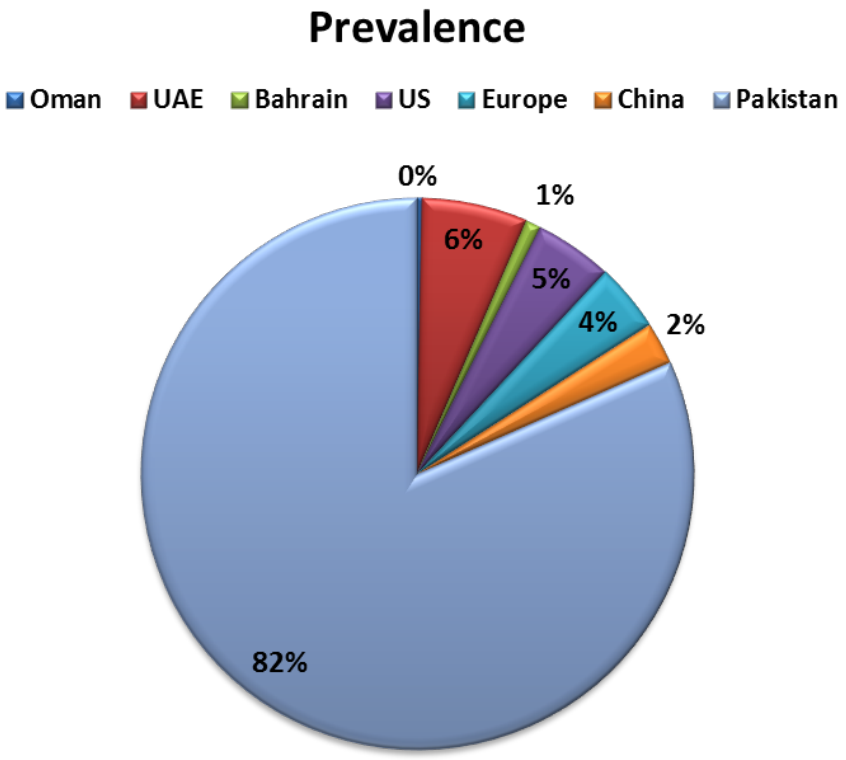

FIGURE 1.2: Prevalence of ASD across world [6], [7].

This is troublesome to know that these costs will become as high as nearly 450 billion dollars in 2025. In this way ASD is affecting the members of family and society also [7], [8].

1.1.3 Etiology of ASD

Although a lot many studies have been conducted to reveal the underlying causes of ASD, but these are poorly understood to date. The theories about etiology of ASD are changing throughout many past years. But nowadays, ASD is thought to be resultant of multiple factors like genetic, epigenetics and environmental factors [3]. In many studies it has been demonstrated that if there is an ASD child in a family then the risk of having another child with ASD is increased up to 25 times as compared to other families. This shows that ASD has strong genetic basis. It has been found that ten to twenty percent of affected individuals have genetic variations.

The environmental factors have also observed to play important roles in increasing the risk of ASD, these factors comprises of vaccination, exposure to pesticides, food habits, infections, use of antibiotics [9],[10]. This is also worthy to note that microbes in gut may have some significance in aggravating the risk for developing ASD. There are many researches that showed the presence of gut disorders in ASD individuals such as "leaky gut". There are also data available on role of gut microbial entities in different neuro-developmental disorders [11].

1.2 Gut Microbiota

All the microbes that are present in gastrointestinal tract of human, along-with the collection of genes of these microbes is termed as "gut microbiome" [12]. The microbiota starts to develop initially just after birth and its diversity in early life depends on factors like mode of delivery and feeding methods. In starting years of life, Lactobacillus and Bifidobacterium are present in more numbers, but the composition does not remain same and changes continuously during initial life stage. Age reaching at 3, this collection of microbes becomes stable and can be said as "adult-like" microbiota. The major proportion of gut microbiota of a healthy person consists of Bacteroidetes while the other portion consists of members of phyla Actinobacteria, Verrucomicrobia and Proteobacteria [13], [11].

There are many significant functions carried out by gut microbiota in our body such as metabolism, digestive system, absorption of nutrients from food, production of vitamins and some neurological roles. There is a huge data present on the communication of gut microbiota and brain that can occur in both directions and is known as "microbiota-gut-brain axis" [14],[15]. It has been shown by researches conducted on mouse models that there is involvement of gut microbiota in brain's functionality and results into altered behaviors [16]. The neural actions occurring in nervous system can be affected by the gut microbial products having neuro-active nature for example dopamine produced by Serratia, GABA from Bifidobacteria and these products enter into blood circulatory system [11],[17].

The products of microbiota such as short chain fatty acids can change the permeability of intestinal membrane and also of blood brain barrier [18]. The change in the permeability of barriers leads to movement of gut microbial metabolites in to the circulation and results in to dysbiosis as experiments have been performed in animal models. It has been proven by animal studies that the changes in number and diversity of microbiota in gut results in to change in behavior in animals. There is also evidence present about if the gut microbiota is changed, then it can also cause good behavioral changes [19], [20].

1.3 Interaction between Gut Microbiota and ASD

Due to the multifactorial pathophysiology of Autism, it is necessary to find out the involvement of these factors in ASD development. As there are number of Autism cases that had gastrointestinal issues, shows that the Autism had some physiological background problems also. In this way if we have to treat this disorder it is must to identify the mechanisms involved in gut-microbiota interaction with brain and causing ASD. There are many reports showing the presence of gut disorders like Crohn's disorder in ASD individuals that is a very painful disease and when present with ASD it is termed as "autistic enterocolitis". It is a well-known saying that "fix your gut, fix your brain" and it really applies to the situation that changing gut microbiota can change gut brain communication which results in altered health condition of nervous system [21],[22].

In numerous studies the association between ASD and gut microbiota has been reported in many ways. Wang et al. found that the GI symptoms are frequently present in autistic children. These include gastrointestinal syndromes like constipation was 20% more in ASD individuals in comparison to their normal siblings whereas diarrhea was 19% higher in them as compared to their normal siblings [23]. Two other meta-analysis demonstrated similar results [24],[25]. Autistic individuals who had gastrointestinal disorders may also experience behavioral problems like

anxiety and aggression [26]. There is a lot of evidence that shows strong association between gut microbiota and ASD by affecting immune system and metabolism [27],[28]. A study presented that ASD patients showed 36.7% increased intestinal permeability, relatives of ASD patients showed 21.2% increased permeability while control children showed 4.8% [29].

This increase in intestinal permeability leads to increased antigenic load. After this, there is an increase in lymphocytes and cytokines like "interleukin-1β", "IL-6", "interferon-γ" and "tumor necrosis factor-α". These move into brain by crossing blood brain barrier. Interleukin-1β and TNF-α after moving into brain produce immune responses in the brain by binding to the brain endothelial cells [30],[31],[32].

It has been observed that in ASD patients and animal models of ASD there is change in composition of microbiota residing in gut [29]. It has been found Hsio et al. that in mouse models bacterial species of Porphyromonadaceae, Bacteroidales, and Lachnospiraceae were higher in number in ASD models whereas normal mouse have the species belonging to Ruminococcaceae and Alcaligenaceae in higher number [33]. It has been reported that children who were autistic have less diverse gut microbiota and they had decreased numbers of Bifidobacterium and Firmicutes and had increased number of Lactobacillus, Clostridium, Bacteroidetes, Desulfovibrio, Caloramator and Sarcina [34]. Autistic children who had gastro intestinal problems have some genera in lower numbers , these genera include Prevotella, Coprococcus, and unclassified Veillonellaceae as compared to normal healthy children [35]. It is also reported that fecal samples of ASD children presented increased levels of *Clostridium histolyticum* group in comparison to fecal samples of normal children [36]. *Clostridium histolyticum* produces neurotoxins that led to systemic effects. If the level of clostridium is reduced by some means it results in major improvements in ASD patients [37].

1.4 Aims and Objectives

Gut microbiota can be one of the etiological agents of ASD because gut dysbiosis is seen in majority of ASD patients. The mechanisms behind gut microbiota involvement in development of ASD, is still ambiguous. An in silico methodology has been designed by exploiting physico-chemical, genetic and functional data of gut microbial metabolites in the form of protein–protein interactions, metabolic pathways and functional annotations, to figure out the role of microbiota in onset of ASD.

1.4.1 Problem Statement

Among various risk factors of ASD, microbiota is considered an important one, but how it contributes to ASD is still unclear.

1.4.2 Proposed Solution

An interaction between gut microbial metabolites and ASD genes could reveal possible mechanisms involved in onset of ASD.

1.4.3 Aim of Study

To figure out the role of gut microbiota in onset of Autism spectrum disorders (ASD), the interaction of metabolites produced by gut microbiota with genes involved in ASD will be analyzed.

1.5 Objectives

To achieve the aim of study, objectives are defined as:

1. Identification of metabolites produced by microbiota in human gut.

2. Prioritization of gut metabolites by analyzing genetic connections between gut microbial metabolites and ASD.

3. Functional analysis of gut metabolites to reveal possible mechanisms involved in onset of ASD.

1.6 Scope

This study is designed to analyze the interaction of gut microbiota in development of ASD. The contribution of this study is the identification of the microbial metabolites present in gut that are playing role in ASD. This study provides the exploration of ASD etiology that will enable the researchers to find out the possibilities of treatment of this disorder. Because of the in silico nature of this study, there is a large amount of knowledge generated by this study that can be utilized by other researchers in biomedical field to find out the mechanisms behind the role of gut microbiota in many other disorders.

Chapter 2

Literature Review

To achieve the aim of study, extensive literature search has been performed to understand onset, etiology, diagnosis, risk factors, and involvement of gut microbiota in ASD. It also covers the available tools for finding out genes associated with ASD, gut metabolites, interaction between gut metabolites and ASD associated genes.

2.1 Autism Spectrum disorders

Autism spectrum disorders, is defined as group of neurological disorders related to social behavior, issues in communication and cognitive abilities. The affected individuals of Autism spectrum disorder face problems in showing interaction with people. They also have interest in a small set of activities and they present a specific behavior for most of the times. The characteristics of the disease can be clearly observed in infants to children of three years. The symptoms of ASD are lack of eye contact with others, inability to control their emotions, face problems in understanding other people 's emotions and show interest a narrow set of activities [2],[3].

2.2 Classification of ASD

The word "Autism" was coined by Paul Eugen Blueler in 1912 for behaviors present in schizophrenia. Hans Asperger used term "autistic" for psychology of child [2]. In DSM-IV autism was categorized as one of the "pervasive developmental disorders". These five disorders are Asperger's syndrome, child disintegrate disorder, autistic disorder, "Rett's disorder", "pervasive developmental disorder-not otherwise specified (PDD-NOS)" [38] . In order to meet changes made by disease community in defining symptoms of ASD, the 5th edition of "Mental disorders diagnostic and statistical manual" included the five above mentioned disorders as sub categories of ASD. According to this manual, autistic patients have problems related to social behavior and they show repetition in behavior. It is also defined in this manual that if there are three symptoms related to communication, two symptoms related to limited behaviors and presence of abnormal type of reaction to sensory stimuli [38]. The children in which repetition of behaviors was not observed but the other two problems existed; these were said to having social communication disorder. The diagnosis of ASD should be done with care that the presence of attention deficit hyperactivity disorder depression and anxiety should not be mixed with ASD [38],[39].The symptoms observed in individuals of different age groups having ASD are described in following table 2.1.

Table 2.1: Symptoms of ASD in different age groups [3].

Communication impairment	Children show delayed speech and some have no speech at all
	Lack of non-verbal communication like pointing towards something
Social impairment	Show no response or less response towards others gestures
	Cannot pretend roles in play

Table 2.1 continued from previous page

	Show little interest in playing with peers
	Cannot start any task
	Unable to share happiness with others
Impairments of behaviors	Repeated hand movements
	Show monotonous behaviors
	Show repetition of activities like switching lights
Schooling children	
Communication impairment	Delay in developing language
	They often show echolalia
	They use uncommon vocabulary as compared to their age group children
Social impairment	aggressive behavior while playing in group
	They do not behave according to norms of class room
Impairments of behaviors	They have no creativity and imagination in playing
	They are unable to accept changes
	They show unique responses towards light, sound etc.
ASD Adolescents	

Table 2.1 continued from previous page

Impairments of communication	In spite of having knowledge of grammar and vocabulary, they have issues in fluent communication
	They cannot have a bi-directional conversation because sometimes they are quiet for long time and sometimes they talk excessively on topics of their own interest
	They lack the ability to use proper style of communication in different social situations
	They show repetition of phrases while talking
	They are unable to understand sarcasm
	They show unusual facial expression and gestures
Social problems	They feel difficulty in making friends of sameage
	They show different behaviors as compares to the other members of their age and social group

Table 2.1 continued from previous page

	They lack knowledge of personal space of others, but are very much reluctant to their own space
Restricted behavior	They show interest in a restricted set of activities/hobbies
	They are happy to follow rigid routines and cannot accept change in routine
	Due to lack of imagination, they show problems in activities like writing, future planning
	Show unique responses to sensory stimuli like smell, taste, sound, touch etc.

2.3 Prevalence of ASD

The prevalence of autism is different across the world, but according to an estimate it is present in 1 to 2 percent population in overall the world [4]. From last twenty years, there has been observed an increase in rate of autism. The National Health Center for Health Statistics measured the autism rate in 2016 and found that it occurs one child among every thirty six children [5].

2.3.1 World-wide Prevalence

Prevalence of autism is different across the world, according to an estimate US has autism rate as 21.6 per 10,000 while Europe recorded it as 18.75 per 10,000

and china showed an average of 11.6 per 10,000 [6]. Gulf Cooperation Council (GCC) countries are facing continuous increase in rate of ASD. In Oman ASD is recorded as 1.4 out of 10,000, while in UAE it is as 29 out of 10,000 and 4.3 out of 10,000 in Bahrain. A study conducted in 2007 showed that in Saudi Arabia ASD prevalence is 1out of 167 persons [7]. The prevalence of ASD across the world is given in figure 2.1.

FIGURE 2.1: World-Wide Prevalence of ASD

2.3.2 Prevalence in Pakistan

In Pakistan there is very low focus on mental health services as only a few teams that work mainly in Punjab and Sindh. Whereas in the other two provinces lack such services. The number of epidemiological surveys conducted to investigate the rate of disorders related to nervous system in children show difficulty in learning is very low[40]. A survey conducted by Hussein et al. in 2011 revealed that in Karachi city schools the occurrence of emotional and behavioral disorders was nearly 17 million in children of age from 5 to 11. A study that included nearly six thousand children showed that 6.5 percent children had "mild mental retardation" whereas 1.9 percent showed "severe mental retardation". Another study found that nearly 16 in 1,000 children of age between 3 and 9 are facing severe mental retardation. A study conducted on occurrence of autism found an increased rate of autism as 2.57 out of 1,000. There are no country level or province level studies on occurrence of autism in Pakistan, most of the studies done have a small sampling area like a hospital or special schools etc. so it is difficult to generalize the results [41].

TABLE 2.2: Prevalence of ASD in Pakistan

Sampling frame	Sampling area	Sample size	Total number of ASD patients	References
Hospital based	Karachi	290	7(2.4 %)	[42]
Hospital based	Rawalpindi	169	9(5.3 %)	[43]
Hospital based	Karachi	200	9(4.5 %)	[44]
Hospital based	Lahore	1,000	32(3.2 %)	[45]

2.4 Impact of ASD on Public Health

ASD is one of the disorders that has high financial burden ASD costs are estimated in different countries as US showed ASD costs to reach approximately $250 billion per year. It is estimated that these costs will increase over 450 billion dollars per year by 2025. With such an increasing prevalence and costs, ASD has become a financial burden on families as well as society. A study done in Saudi Arabia explored the impact of ASD patients on their families and revealed that autistic child have 59.9% negative effect on family' social life style, 60.4% families were affected economically, relationships of families were affected for 53.5% families, 62.6% autistic children affected the life of his or her siblings and 88.5 % children caused parental distress [7].

2.4.1 Impact on Families

ASD has been found to affect the quality of life of all family members. Because the autistic child faces difficulties in daily activities so it becomes a burden on parents as he or she needs extra care. Banach et al. [46] explored that on diagnosis of autism, parents were in grief of 4% autistic children, while 29% were shocked, 52% parents felt relieved, 10% of parents blamed themselves. Overall parents felt stress because of their child's diagnosis and how their child will adapt to new life style.

It has been found by studies that mothers were in more stress as compared to father of autistic child [7],[8].

The major problem faced by parents of autistic child is that he or she cannot tell them about basic needs. This results in development of aggressive nature in child because the parents are unable to understand what the child wants if he or she is feeling pain, hunger or sad etc. Parents feel it difficult to take their child for visiting their relatives or some public places because outside people are not able to understand child's behavior. In this way parents feel that they are isolated from society because of their autistic child. Autistic child needs extra care and attention which becomes financial burden for the parents as they need to appoint someone for their child's care or they have to quit their own job to take care of their child. Siblings of autistic child sometimes feel embarrassed in front of their friends, they may sometimes feel jealous by their parents' attention towards their autistic sibling and sometimes they got frustrated by their autistic sibling's behavior [7]. A study conducted in Saudi Arabia to find out the impact of autistic children on families. It included 227 families out of which 88.5% claimed that autism treatment centers play a good role in treatment of their child but 31.7% parents sent their children to autism centers regularly. Among the therapies used for autistic children, speech therapy is considered the most common one, whereas behavioral therapy comes second and third is pharmacological therapies. Some families have been reported to use traditional treatment methods like cauterization and roqia. Some parents preferred to treat their child by dietary therapy which includes mostly gluten free diet or sugar free diet [47].

2.5 Neurobiology of Autism

Recently it has been found that neurobiological changes are involved in pathophysiology of ASD, although these findings are being revised many times [2].

2.5.1 Amygdala and ASD

In ASD patients, it has been observed that the frontal lobe and temporal lobe are the most affected parts of brain. In a number of studies it has been proved that amygdala plays role in cognition and ASD. Amygdala's major functions include eye gaze, social behavior, face processing and modulation of memory [48]. The results of tumor in amygdala are quite similar to the neuropathology of ASD. It is involved in receiving visual, auditory, somatosensory and other visceral inputs. The amygdala contains 13 nuclei these belong to three sub groups that are basolateral, superficial and centromedial groups. The basolateral group plays role in higher level of social cognition. This group acts as a bridge between centromedial and superficial groups. It is involved in showing response towards face expression and actions of other people. [49]. It has been showed by many studies that amygdala contains GABAa receptors in high numbers and have many opiate receptors, serotonergic, cholinergic, noradrenergic, dopaminergic cell bodies and pathways [50]. In some reports it has been suggested that there are deficits in amygdala in ASD patients. In post mortem studies it has been proved that there is pathology in amygdala in ASD patients, such as small size of neurons and increase in cell density in three nuclei of amygdala that are cortical, medial and central nuclei. The experimental manipulations of the amygdala in animal studies have shown to produce ASD like symptoms [51].

2.5.2 Prefrontal Cortex and ASD

Frontal lobe is suggested to have role in higher level cognition and ASD. Deficits occurring in frontal lobe leads to dysfunctioning in language, cognition social behavior and emotions. It has been reported that there is an increase in volume of frontal cortex in new-born babies with ASD [52]. There are two divisions of prefrontal cortex that are medial PFC and lateral PFC. Medial PFC is involved in processing of emotions, memory and cognition whereas medial PFC plays role in cognitive control process [53]. The role of medial PFC in social cognition involves

behaviors like understanding own self and others. The medial PFC plays role in fear learning because of its synaptic connections with amygdala. The disturbance of communication between mPFC and amygdala leads to problems in memory processing that proves the role of medial PFC in ASD [54].

2.5.3 Nucleus Accumbens and ASD

Nucleus accumbens is suggested to play role in social reward response in ASD [55]. Dysfunctioning of nucleus accumbens related to reward system leads to depression. Bewernick et al. reported that there are positive effects in patients of depression by NAc-DBS [56]. ScottVan Zeeland et al. found that deficit in ventral striated response in ASD patients when they were given social rewarded learning [57]. In ASD individuals the ventral striatum showed low activity and resulted in loss of social reciprocity [57].

2.6 Diagnosis of ASD

The diagnostic scales used for ASD are needed to be quite accurate because the symptoms and scope of ASD overlaps with many other psychiatric disorders. Diagnostic methods consists of interviews by parents or caregivers, interview of individuals with ASD, direct case assessment and a review of family background in depth for ASD or other psychiatric disorders [38]. The methods that are used commonly to diagnose ASD are listed in table 2.3.

Table 2.3: Diagnostic methods used commonly for ASD

SN	Method	Details	Score	Age	Time	Ref
1.	Developmental, Dimensional, and Diagnostic Interview (3di)	Interview of parents or care givers, it is mostly computer based or by investigators.	0 score shows no evidence while 2 shows definite evidence	Early childhood through adulthood	1.5 to 2 hours	[58]
		It has total 740 questions in which 183 belong to family history, 266 include ASD symptoms and 291 are about any comorbidity				

Table 2.3 continued from previous page

SN	Method	Details	Score	Age	Time	Ref
2.	Childhood autism rating scale (cars)	There are total 15 items that include questions for comparing behavior of a normal child with an ASD child	30 to 37 scores mean “mild to moderate ASD” \ whereas scores from 38 to 60 means “ severe ASD”	Early childhood through adulthood	Not mentioned	[38]
3.	ASD observation for Children (ASD-OC)	There are 45 items that are used for evaluating individual for ASD symptoms such as social impairment , problems in communication etc.	“0” means “ no impairment”, “1” means “ mild impairment” and “2” means “ severe impairment”	Children of age between 3 to 15 years	Not mentioned	[59]

Table 2.3 continued from previous page

SN	Method	Details	Score	Age	Time	Ref
4.	Autism diagnostic interview revised	Interview is taken from parents/ caregivers by investigators. There are total 13 items to investigate behavior according to age such as social behavior, language development , communication, repetitive behaviors.	"0" means no evidence and "3" represents extreme impaired behavior	Children with mental age equal to 2 and adults	2 to 3 hours	[60]

Table 2.3 continued from previous page

SN	Method	Details	Score	Age	Time	Ref
5.	Asper syndrome and high functioning autism diagnostic interview (ASDI)	There are total 20 items of 6 categories.	The definite score in three out of six items.	Childhood through adulthood	15 to 20 mins	[61]

Table 2.3 continued from previous page

SN	Method	Details	Score	Age	Time	Ref
6.	The Diagnostic Interview for Social and Communication Disorders	Interviews of parents or caregivers in semi-structured form.	A dimensional approach is used instead of cut off scores and tries to recognize the social and communication impairment patterns over years	Infants to adults	2 to 3 hours	[62]

2.7 Management Strategies for ASD

When there is a patient having ASD like symptoms, physician suggests some laboratory tests to support the diagnosis. Tests like electroencephalography EEG can help in revealing ASD or other comorbidities that result in selection of therapies and drugs. Neuro imaging techniques like magnetic resonance imaging (MRI) can help in diagnosis of ASD by unveiling changes related to volume loss of deep grey matter that is seen in many ASD individuals [63].

ASD is a long span disorder and there is no treatment for it to date but many drugs are used for managing behaviors related to ASD. It has been suggested by researchers that the early behavioral therapy is the best treatment because it showed improvement in behavior of ASD patients. This therapy is based on language development, social behavior and pretending skills. "Treatment and education of autistic and related communication handicapped children (TEACCH)" and applied behavior analysis are two types of this type of therapies. Families of autistic children are also using alternative treatments like vitamins intake and supplements, detoxification therapy and hyperbaric oxygen therapy in order to decrease the symptoms associated with ASD [64], [63].

2.8 Medications

For the management of symptoms and comorbidities associated with ASD, some pharmacological and non-pharmacological therapies can be used. Although many studies have been done to reveal the disease causing agent but it could not be possible yet. The treatment of ASD to date includes some pharmacological and non-pharmacological therapies like drugs such as anti-psychotic drugs, psychostimulants, anti-depressive drugs, and anti-epileptic mood stabilizers[38].

These drugs may help in controlling symptoms of ASD but they also show side effects in individuals so the must be used only when prescribed by some specialized

person such as a neurologist or psychiatrist. FDA has approved some drugs for the treatment of ASD to cure aggressiveness [65].

Cobalamin is also suggested for the cure of ASD because it is well known for brain and nervous system improvement. Cobalamin has many different types but most important is methyl B12 because it helps in providing energy to brain, Methyl B12 levels have been observed in ASD individuals that these levels are low in them as compared with healthy individuals [66]. Another study presented that injecting methyl B12 produces improvement in behavior of ASD patients as compared to individuals who were not given methyl B12. This study had many limitations like sample size was small but the results are linked to the discovery of the reduced methyl B12 level in brain [66] the medications that are used for ASD treatment are given in table 2.4.

Table 2.4: Medications used for Autism spectrum disorder [38]

Condition	Drugs
Irritability and aggression	Risperidone, aripiprazole, clozapine, haloperidol, sertraline
Aberrant social behavior	Oxytocin, secretin
Hyperactivity and inattention	Methylphenidate, venlafaxine
Repetitive behaviors	Fluoxetine, citalopram, bumetanide
Cognitive disorders	Memantine, rivastigmine
Insomnia	Mirtazapine, melatonin

2.9 Etiology

Although a number of studies have investigated the cause of ASD, but these causes are poorly understood to date. The theories about etiology of ASD are changing throughout many past years. Different theories have been rejected; one of these is issues in child rearing that was considered the cause of ASD onset in children

until 1970s. But nowadays, ASD is thought to be resultant of multiple factors like genetic, epigenetics and environmental factors [3].

2.9.1 Genetic Factors

It has been shown by family studies that during diagnosis of other disorders like tuberous sclerosis, phenylketonuria or congenital infections 10% of the children are found to be ASD patients [67]. In many studies it has been demonstrated that if there is an ASD child in a family then the risk of having another child with ASD is increased up to 25 times as compared to other families. The concordance rate of having ASD for identical twins is 60-90% while it is 0 to 24% in non-identical twins. It has been found that structural variations play their role in increasing risk of ASD [7].

2.9.1.1 Genetic Studies of ASD

As ASD is a multifactorial disorder, so it is quite difficult to find out the exact genetic cause behind it. A number of approaches are needed to find out the loci that are involved in ASD [68]. Although some studies have been done in order to find out the genetic cause of ASD but there are some pros and cons of each study. In these studies mostly the approaches used are cytogenetic studies, DNA microarray analysis. linkage analysis, copy number variation and whole-exome sequencing [7].

2.9.1.2 Cytogenetic Studies

Cytogenetic analysis is the major approaches used in genetic studies in which chromosomal abnormalities are searched. It has been hypothesized that autism is caused by some rare de novo mutations that are not transferred to nest generation. Cytogenetic studies have revealed many candidate genes and chromosomal aberrations related to ASD [7].

2.9.1.3 Copy Number Variation Analysis

CNV comprises of detection of insertions and deletions of DNA fragments of size more than 50 KB and it is one of the latest cytogenetic techniques. According to many studies copy number variations have been found in SHANK2, SYNGAP1 and many other genes. These loci were detected to be affecting ubiquitin pathways in ASD individuals. Genes that are involved in synaptic functions such as NLGN1, NLGN2 and many other genes were also found to have copy number variation [69].

2.9.1.4 Linkage and Association Studies

The aim of linkage studies is to find out the inheritance of ASD associated loci in families of ASD individuals. It has been demonstrated by association studies that alleles and genotype frequencies is different in ASD families and control group families. On the basis of linkage studies some single nucleotide polymorphisms have been identified. Association studies are unable to find out the rare mutations because only susceptible alleles are studied in these studies [70].

2.9.1.5 Microarray Analysis

Microarray analysis is a good option to find out submicroscopic chromosomal abnormalities because of great advance sin microarray technology. Due to this technology, it is possible to detect de novo and rare mutations. For many disorders, whole exome arrays have been developed to identify rare mutations. Whole exome sequencing helps in detecting uncommon mutations when linkage studies cannot be performed. In order to develop better understanding of causes of ASD studies have exploited gene expression data of some genes. In last few years trials have been made in order to find the diagnostic biomarkers for autism but yet no such a useful biomarker has been identified. In Saudi Arabia a study had been performed in 2017 using whole exome sequencing in19 trios subjects revealed that 17 trios had 47 rare variants from which 38 were first time revealed. Out of these variants a large number of variants were “X-linked” or “autosomal recessive” [7].

2.9.1.6 Genome-Wide Association Studies

Studies have been performed in order to determine important candidate genes, one type of these studies is GWAS which has led to provide a list of important candidate genes but the pathophysiological process of ASD is not clear yet. A study conducted by Wang et al. [71] and Glessner et al. [72] comprised 3000 Europeans from nearly seven hundred families and consisted of 6491 control cases. This study reported six SNPs between CDH9 AND CDH10. Among these SNPs the most significant SNP was rs4307059 in chromosome 5. In another study conducted by Anney et al. [73] it has been confirmed that SNP rs4141463 was found to be strongly associated with ASD. Cho et al. [74] performed a genome wide study to evaluate forty two Korean ASD individuals and candidate SNPs were found on chromosome 11. These SNPs were found to be associated with language issues in ASD patients.

2.9.1.7 Environmental Factors

By a number of earlier studies it has been found that 80 to 90% of ASD cases are a result of genetic factors with some environmental factors. But nowadays many latest studies showed that 40 to 50% ASD cases involve environmental factors. It has been demonstrated by studies conducted on twins that both of genetic as well as environmental factors play role in ASD [8].

2.9.1.8 Parental Age

Wu et al. conducted a study based on meta-analysis and analyzed 27 studies that showed association of parental age with autism development. This analysis showed that per 10 years increase in maternal age or paternal age results in 18 to 21% increase in the risk of having ASD child [8].

2.9.1.9 Medication Use During Pregnancy

A study conducted by Gentile [75] found the association between Valproate usage by mother in pregnancy to autistic child. But it has many controversies about the use of anti-depressants by pregnant women and ASD. Case control studies demonstrated a 50 % increase in risk of having autistic child in pregnant who took selective serotonin reuptake inhibitors (SSRIs). It was suggested by another study that the association between ASD and SSRIs is dependent on some not known factors [76].

2.9.1.10 Smoking and Alcohol Use in Pregnancy

Although maternal smoking and use of alcohol in pregnancy has been found to produce negative effects on child's health but according to studies the association between ASD and these factors have not been established. In regard of this, a meta-analysis based study done by Rosen et al. analyzed fifteen studies and revealed that no evidence of increase in risk of autistic child for mother who did smoking or took alcohol in pregnancy [76].

2.9.1.11 Vaccination

The relationship between vaccination and development of ASD has been investigated by many researchers but it is controversial that either it has any link with development of ASD or not. Taylor et al. [77] performed a meta-analysis in order to check the association of ASD and vaccination in children, but it found no evidence of having increased risk for onset of ASD. In a report Wei et al. [78] that vaccination produces symptoms of depression because they contain aluminum nano-particles and these particles trigger inflammation in brain. Gherardi et al. [79] reported vaccine that contain aluminum that goes to brain but it is not sure that either vaccination is associated with ASD or not.

2.9.1.12 Gut Microbiota

Gut microbiota comprises 3 to 4 million genes collectively that is nearly 100 to 150 times more as compared to human genome. A number of genes present in gut microbiota express to form proteins and metabolites. These metabolites act in the local environment and can act into the circulation. After moving into the circulation these metabolites may go to distant organs such as brain. By reaching these distant organs, these microbial products can act as ligands and can change host genes expression level. These metabolites are also reported to enter into metabolic pathways and can change their activities. [80].

2.10 ASD and Gut Microbiota

In numerous studies the association between ASD and gut microbiota has been reported in many ways. Wang et al. found that GI symptoms are frequently present in autistic individuals. These include gastrointestinal syndromes like constipation twenty percent higher in ASD individuals than their normal siblings whereas diarrhea was 19% higher in them as compared to their normal siblings [23]. Two other meta-analysis demonstrated similar results [24],[25]. Autistic individuals who have gastrointestinal disorders may also experience behavioral problems like anxiety and aggression [26]. There is a lot of evidence that shows strong association between gut microbiota band ASD by affecting immune system and metabolism [27],[28]. A study presented that ASD patients showed 36.7% increased intestinal permeability, relatives of ASD patients showed 21.2% increased permeability while control children showed 4.8% [29].

This increase in intestinal permeability leads to increased antigenic load. After this there is an increase in lymphocytes and cytokines. These move into brain by crossing blood brain barrier. Interleukin-1β and TNF-α after moving into brain produce immune responses in the brain by binding to the brain endothelial cells [30],[31],[32].

It is seen in ASD patients and proved in animal models of ASD there is change in composition of microbiota residing in gut [29]. It has been found Hsio et al. that in mouse models bacterial species of Porphyromonadaceae, Prevotellaceae, and two others were higher in number in ASD models whereas normal mouse have the species belonging to Ruminococcaceae, Erysipelotrichaceae, and Alcaligenaceae in higher number [33]. It has been reported that autistic children have low diversity of gut microbiota and they have decreased numbers of Bifidobacterium and Firmicutes and have increased number of Lactobacillus, Clostridium and some other genera [34]. It is also reported that fecal samples of ASD children presented increased levels of Clostridium histolyticum group in comparison to fecal samples of normal children [36]. Clostridium histolyticum produces neurotoxins that led to systemic effects. If the level of clostridium is reduced by some means it results in major improvements in ASD patients [37].

2.10.1 Pathways of Microbiota-Gut-Brain-Communication

There are many mechanisms suggested by using which gut microbiota and its products can led to changes in physiological and pathological pathways. These mechanisms consist of three major pathways that are vagal pathway, immune pathway and biochemical pathway hese pathways are shown in figure 2.2. The communication between brain and gut majorly takes place through vagus nerve. Gut microbiota or their metabolites stimulate intestinal afferent fibers and this stimulation is then passed over to nucleus tractus solitarius, from there it moves to thalamus, amygdala, hypothalamus and periaqueductal grey. When the vagus nerve is electrically stimulated by microbiota there is a change in amount of neurotransmitters such as 5-HT, glutamate and γ-aminobutyric acid (GABA) that is observed in both mouse and human brain. It has been shown in mouse model studies that the depression present due to immune system problems through vagal pathway can be eliminated by vagotomy [81].

Metabolites produced by gut microbiota can change emotional behavior by three pathways; first is the activation of vagus nerve, second is the stimulation of immune

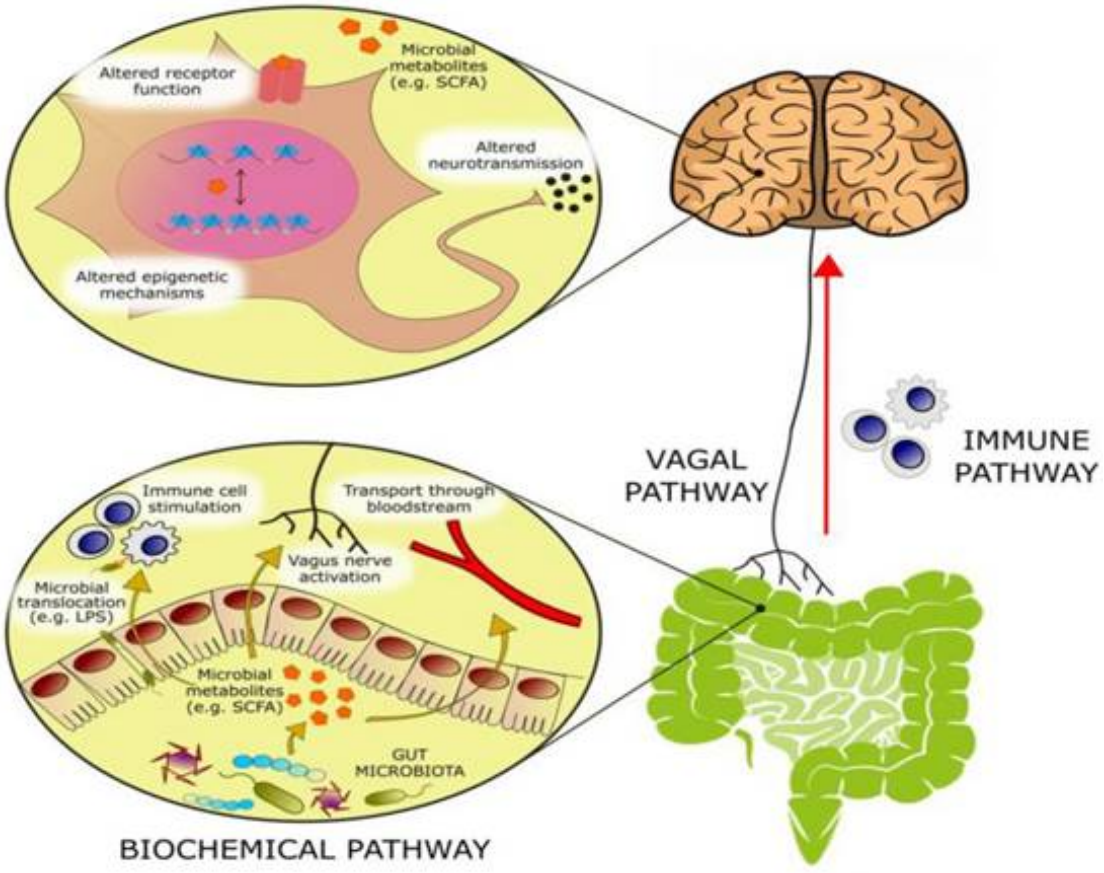

FIGURE 2.2: Pathways by which human gut microbiota interacts with brain

cells of mucosa or of circulatory system, third is through biochemical interactions of metabolites with receptors on neurons

The gut microbiota talks with brain by the help of immune system. In general immune system plays many physiological roles such as sleep and memory making are functions of central immune cells and normal range of inflammatory mediators, but if there is any neural inflammation then it have adverse effects on brain health and can led to neuropsychiatric disorders. In a normal healthy individual it has been suggested that gut microbiota influences host's immune system greatly, as in childhood it is associated with normal growth of immune cells such as astrocytes and microglia [82]. Whereas in adults it leads to low level inflammation because cytokine release from intestinal macrophages and T cells stimulated by bacterial antigens residing in gut. It has been shown by reports that peptidoglycan that are component of bacterial cell wall are responsible for stimulation of innate immune

system and changing behavior by reaching brain and activating pattern recognition receptors [83].

Lipopolysaccharides are found to cause release of cytokines IL-18. It has been observed that depression and cognition symptoms are faced by healthy individuals when LPS are administered to them [84]. Most importantly, the movement of LPS to brain is controlled by propionate that is a gut metabolite having the ability to increase blood brain barrier permeability [85]. The transportation of bacteria and lipopolysaccharides into circulatory system may result from leaky gut (gut with increased permeability of intestinal wall) which can be caused by factors like stress and Western diet [86].

The third pathway of communication is through direct biochemical signaling with help of gut microbial products. The microbial products include metabolites produced through fermentation of food by bacteria. These metabolites can enter in to circulatory system where they can show interaction with different enzymes and receptors, resulting in to physiological and pathological changes. For example it is proved by evidence that acetate, a microbial metabolite, can produce change in neural functionality. Some microbial metabolites can act as neurotransmitters such as SCFAs, choline metabolites, vitamins and lactate can affect neural processes and dysregulation of these metabolites may lead to nervous system disorders [80].

2.10.2 Gut Microbial Metabolites

Gut microbiota is reported to play role in ASD with the help of metabolites it produces. Metabolites produce by gut microbiota like SCFAs, free amino acids (FFAs) and phenol compounds are known to produce ASD behaviors by moving to brain through vagus nerve [87]. SCFAs play beneficial roles like energy homeostasis, reduced body weight and reducing risk of colon cancer [88].

It has been shown by many studies SCFAs are involved in pathology of ASD. Wang et al. [89] found the role of SCFAs in autism and reported that autistic

children had SCFAs and ammonia in large numbers in fecal samples as compared to normal ones. One of these SCFAs, propionic acid is able to enter in to brain by crossing blood brain barrier and produced ASD symptoms [90]. Propionic acid has been proved to produce impairments in social behavior, abnormal repetitive movements by changing levels of dopamine and serotonin in brain [91]. Another metabolite, butyrate, that is also one of the SCFAs, can alter the level of dopamine, epinephrine and norepinephrine by changing tyrosine hydroxylase gene expression [92]. Butyrate also shows anti-inflammatory effects [93]. Free amino acids produced by gut microbiota by protein hydrolysis, are also reported to play role in ASD. It has been found by De Angelis et al. [34] that free amino acids were present in large concentration in fecal samples of autistic children.

2.11 Comorbid Disorders

It has been observed that 75% of ASD patients have some other comorbidity like other psychiatric disorders. As a result of these comorbidities patients have to face more impairment in behavior. In this type of cases treatment becomes more difficult. The comorbidities that are mostly present with ASD are discussed below.

2.11.1 ADHD

Attention deficit hyperactivity disorder is frequently seen in ASD patients. It has been demonstrated by studies that 30 to 50% of ASD patients have ADHD, whereas patients having ADHD also display ASD like symptoms [94],[95]. The common symptoms of both of these disorders are lack of attention, impulsive behavior and hyperactivity. These disorders have genetic basis and are found more in boys as compared to girls. These disorders become a hindrance in carrying out daily activities [96].

2.11.2 Depression

Depression is present in many ASD patients, but it is quite difficult to diagnose depression in ASD patients because of the co-occurring ASD symptoms [97]. It has been found that 10 to 50% ASD patients suffer from depression [98]. ASD children face longer periods of depression as compared to depression in non-ASD children [99]. According to studies, depression is more prevalent in ASD adults in comparison to depression in healthy adults or adults having disorders other than ASD [100].

2.11.3 Anxiety Disorders

Anxiety disorders are seen in 80% of autistic children [98]. Among anxiety disorders the most prevalent one in ASD is Separation anxiety disorder that is present in 38% ASD patients, after this obsessive compulsive disorder is present in 37% ASD patients, generalized anxiety disorder are in 35% ASD patients and social phobia is present in 30% of ASD patients [101]. Patients who are autistic and have obsessive compulsive disorder show repetitive behaviors like tapping, counting and repetition of words etc. [102].

2.11.4 Bipolar Disorder

Bipolar disorder often occurs in autistic adolescents. It has been found that 30% of ASD patients also have bipolar disorder [103].It has been shown by another research that bipolar disorder was highly present in 44 ASD patients [104]. The diagnosis of bipolar disorder in ASD patients is critical otherwise treatment of ASD will not be possible.

2.11.5 Tourette Syndrome

Tourette syndrome has symptoms like disturbing non- rhythmic movements due to the affected motor neurons and presence of vocal ties. It is well documented that Tourette syndrome rates are higher in ASD patients. A study found that Tourette syndrome is present in 22% of autistic individuals [105]. It is suggested that comorbidity between Tourette syndrome and ASD means that are some common genetic basis for both disorders [106].

Chapter 3

Materials and Methods

This study is designed to find out the mechanisms by which gut microbiota is involved in causing ASD the effort is made of identify metabolites produced in gut by microbes and will check their relation with ASD on genetic and functional level. To attain this goal, an in silico methodology is defined by exploiting physico-chemical, genetic and functional data of metabolites such as protein–protein interactions, metabolic pathways and annotations.

The analysis of gut microbial metabolites are performed at two levels, genetic level and functional level. Analysis at genetic level provides an insight to genetic connections between gut microbial metabolites and ASD, whereas analysis at functional level is a multi-step process which includes physico-chemical properties evaluation, gene ontology and pathway analysis of metabolites. Validation of predicted ASD associated metabolites are done by using metabolites already reported to be associated with ASD through literature search for ASD associated gut microbial metabolites. For all these steps we will use databases and tools that are available publically. These include AutismKB 2.0 for genetics of ASD, human metabolome database, and other tools, whose details are discussed in each step. The steps performed to analyze the connections between gut microbiome and ASD are shown in figure 3.1.

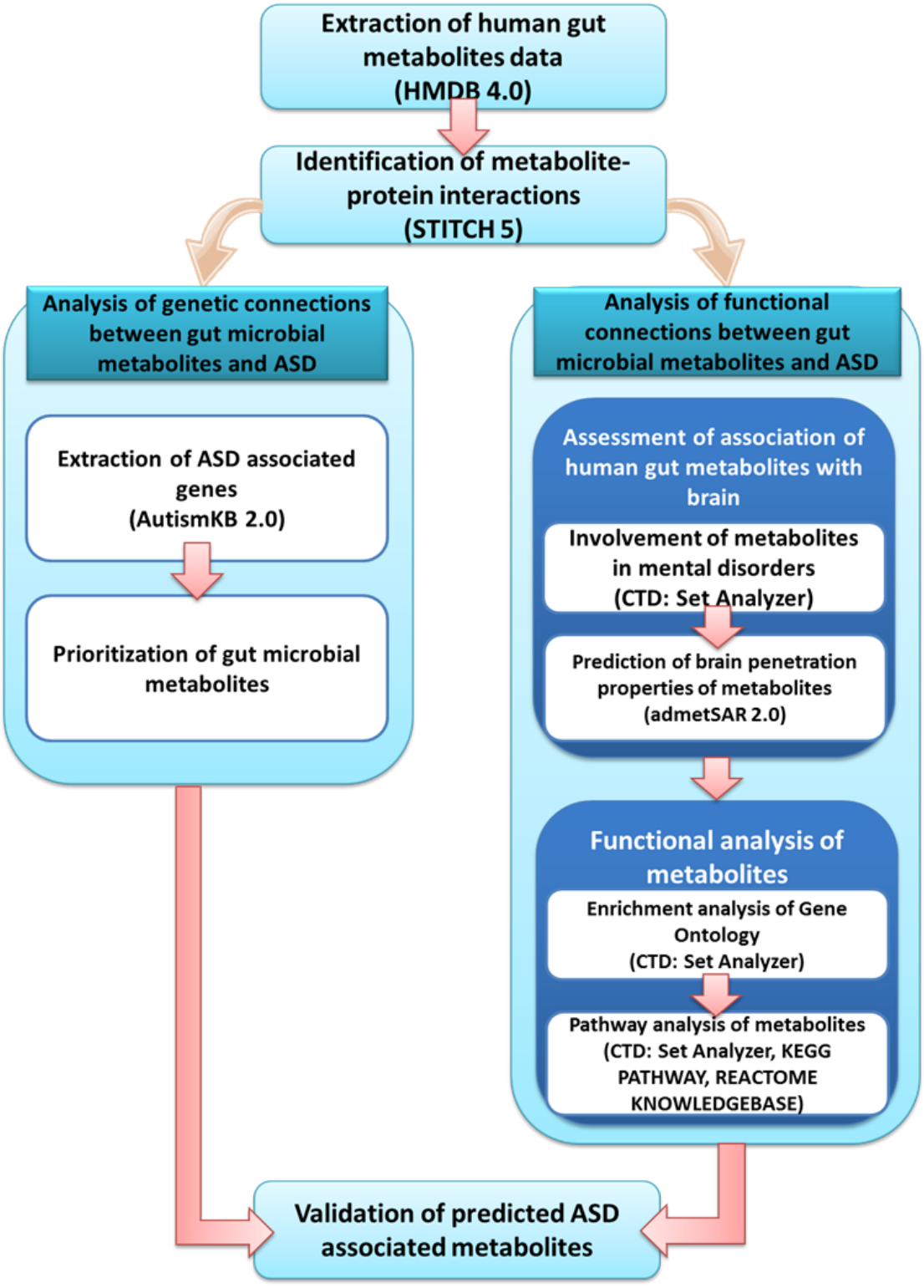

FIGURE 3.1: Steps performed to analyze the connections between gut microbiota and ASD

3.1 Extraction of Human Gut Metabolites Data

Gut microbiota influences host metabolism by producing metabolites through fermentation of dietary components. These metabolites play role in shaping gut microbiota host's physiological and pathological activities. The metabolites can communicate with brain by three mechanisms. Metabolites produced by gut microbiota can change emotional behavior by three pathways; first is the activation of vagus nerve, second is the stimulation of immune cells of mucosa or of circulatory system, third is through biochemical interactions of metabolites with receptors on neurons [80].

For retrieving gut metabolite data Human Metabolome database HMDB 4.0 (available at http://www.hmdb.ca/) was used, that is the most comprehensive database of human metabolites and is considered the standard metabolomics resource because it consists of 114,160 small molecules human metabolites along with their details such as their roles in body, their associations with disease if any , chemical reactions and metabolic pathways. In this study, search is performed for metabolites produced in human gut by microbiota after applying filter of microbial origin. The HMDB database provides search by using text, id, location, origin and the result consists of name, HMDB ID, smiles structure, formula, average mass, monoisotopic mass and bio specimen location of metabolites. [11],[107] .

3.2 Identification of Metabolite-Protein Interactions

In order to reveal the role of identified human gut metabolites, proteins that show interaction with these metabolites are searched. For this purpose STITCH 5 "Search tool for interaction of chemicals" is used (available at http://stitch.

embl.de/). This is a strong resource because it contains data about 0.5 million molecules belonging to more than two thousand organisms. The information present in this database comes from three sources, metabolic pathways, experiments and drug target interactions. STITCH 5 predicts relations between chemicals by using text mining and by checking similarity of chemical structures. Information from STRING regarding genomic context and gene fusion is used for prediction of protein-protein interactions. Interaction between chemicals is predicted from MeSH pharmacological actions [107],[108]. Three types of interaction scores are provide by STITCH 5, where one score is based on the experimental data sources such as PDSP Ki and PDB. The second score is based on data from KEGG and Reactome. The third one is based on data from text mining from OMIM and MEDLINE. It also provides a combined score that is calculated by using three scores by Bayesian scoring scheme.

In this study, genes associated with each of the gut metabolite are obtained from STITCH 5; by using any one input method out of search by name or structure or sequences. The result of this tool consists of three types of interaction scores that are, "experimentally determined interaction score", database annotated score, "text mining interaction score" and combined score. The tool gives a combined score that ranges from 0 to 1 where 0 means "no interaction" whereas 0.4 means "medium confidence" and 0.7 is the default cut off value for highly confident interactions [109].

3.3 Analysis of Connections Between Gut Metabolites and ASD at Genetic Level

It is hypothesized that if any metabolite is showing interaction with ASD associated genes this shows that this metabolite is playing role in ASD. The gut metabolites are filtered which are showing sharing of genes with ASD. For this purpose, ASD-associated genes are obtained from AutismKB 2.0 as mentioned below.

3.3.1 Extraction of ASD Genes

Autism spectrum disorder (ASD) is one of the neurodevelopmental disorders with strong genetic basis. It has been demonstrated by family and twin studies that constitute a major etiological factor for ASD. Mutations of both types inheritable and de novo have been observed to play their role in pathophysiology of autism [4],[7].

For getting the genes involved in ASD, AutismKB 2.0 is used because it a large resource containing comprehensive genetic evidence about ASD. It consists of total 1,379 ASD associated genes out of which 1,280 genes are non-syndromic and 99 are syndromic. In this database, information about genetic variations such as copy number variations, insertion and deletions, and linkage regions is also present. Out of the total genes, 171 genes are designated as core data set because of having high confidence score. These 171 genes consist of 99 syndromic and 109 non-syndromic autism genes. This core data set of genes are retrieved for analysis [4].

3.3.2 Prioritization of Gut Microbial Metabolites on the Basis of Shared Genes with ASD

To check if any genetic connection exists between gut microbial metabolites and ASD, for all metabolites it is searched that how many genes are common between metabolite interacting genes and ASD related genes. This will provide the metabolites that have any genetic connection with ASD.

3.4 Analysis of Connections between Gut Metabolites and ASD at Functional Level

To evaluate the metabolites that they have any connection with ASD at functional level, two approaches used. First approach is used to evaluate association of

each gut metabolite with brain by finding involvement of metabolites' interacting proteins in any cognitive disorder along with having physico-chemical properties that facilitates in crossing blood brain barrier while second approach is based on gene ontology and pathway analysis.

3.4.1 Assessment of Association of Human Gut Metabolites with Brain

To evaluate association of each gut metabolite with brain, involvement of metabolites interacting proteins in any cognitive disorder works as a signal that the metabolite can be involved in affecting some brain function. In addition to this, blood brain barrier crossing ability of metabolites are checked that will provide an insight that a metabolite can be responsible of entering and damaging brain in some way.

3.4.1.1 Involvement of Metabolites in Mental Disorders

To filter out the metabolites, metabolites that have some association with brain are separated. For finding the metabolites that are involved in mental disorders, inference of diseases are done for all gut microbial metabolites. This inference of diseases is performed by using Set Analyzer a tool of Comparative Toxicogenomics Database (CTD) because it easily available. It consists of information regarding gene and protein association with disease. This tool is used to perform enrichment analysis of set of genes or chemicals. It predicts diseases for set of genes using MEDIC disease vocabulary that is developed by combining MeSH and OMIM databases. Input can be given in the form of genes ids. This provides all the diseases that are found to be enriched among the provided gene set where enriched means that more genes out of the given set annotated to that disease is greater than the proportion of genes out of whole genome annotated to same disease. By applying Bonferroni correction, corrected P-value is used for disease enrichment analysis as a significance threshold.

In this tool, enrichment analysis is performed for each metabolite by using interacting genes given by STITCH 5 as input data set, and a significance threshold that is p-value, was selected for enrichment analysis. The output of this tool consists of disease category, p-value, corrected p-value, number of annotated genes and genome frequency [110],[111]. By this analysis metabolites involved in some nervous system and mental disorders are filtered for further analysis.

3.4.1.2 Prediction of Brain Penetration Properties of Metabolites

Brain is separated from blood with the help of a separation known as blood brain barrier. It is composed of network of cell like endothelial cells, pericytes, astroglia, macrophages and basal lamina [112]. Blood brain barrier crossing ability of metabolites are analyzed to determine the possible effects of metabolites on brain function. The diffusion of any chemical across blood brain barrier depends on the strength of the inter-molecular forces between the chemical and water molecules [113].

This ability of metabolites to cross BBB is predicted by using webserver admetSAR 2.0, that utilizes "quantitative structure activity relationship (QSAR) models" to analyze absorption, distribution and other properties of chemicals. This server is selected because it contains over 200,000 ADMET data points for 96000 unique compounds and it also provides a user-friendly interface. Smiles structures of metabolites are used to predict BBB permeability for metabolites. The result of admetSAR 2.0 consists of forty seven models that are intestinal absorption, blood–brain barrier penetration, hERG inhibitors, and many others [114].

3.4.1.3 Filtration of Brain Associated Human Gut Microbial Metabolites

Metabolites that are found to have association with any mental or nervous system disorder and are also be predicted to cross BBB are considered as brain associated human gut microbial metabolites and are used for further analysis.

3.5 Functional Analysis of Metabolites

Functional analysis of metabolites is important in order to categorize metabolites as ASD associated or not. It is done by functional enrichment analysis and Pathway analysis of metabolites, which will help us to understand the role of metabolite in ASD.

3.5.1 Functional Enrichment Analysis of Metabolites

This analysis provides the functional annotation of a set of compounds, so it gives insights into the overrepresented functions of the compounds. These years functional enrichment analysis is frequently used in the field of metabolomics for the analysis of metabolites. This analysis was performed on the brain associated gut metabolites, by using two tools one is Set analyzer of Comparative Toxicogenomics Database [115].

3.5.1.1 Enrichment Analysis of Gene Ontology of Brain Associated Human Gut Metabolites

For performing enrichment analysis, a tool of Comparative Toxicogenomics Database is used. It provides the enrichment analysis based on gene ontology for metabolites, where gene ontology (GO) are the terms of describing functions of protein in three different categories named as "cellular component", "molecular function" and "biological process" [111]. CTD is a powerful database that is used for analysis of metabolites using proteins that have shown interaction with each metabolite. A significance threshold that is p value can be set. The default p value of the tool is 0.01. This analysis is applied on ASD genes and metabolites' interacting proteins, after which metabolites are filtered, which will have common GO terms with ASD GO terms. The result of this analysis consists of gene ontology type, "GO term name", p value, number of genes annotated, symbols of annotated genes and genome frequency [110].

3.5.2 Pathway Analysis of Metabolites

Pathway analysis of metabolites is performed to analyze the biological content of metabolites filtered by the previous steps. This analysis is performed using set analyzer of CTD, KEGG and Reactome.

Enriched pathways among the interacting genes for each metabolite is predicted by set analyzer a tool of Comparative Toxicogenomics database because it is available publically and contains information about the interaction of proteins and diseases. It predicts the pathways and call them as enriched if a greater proportion of given gene set is annotated to that pathway as compared to the proportion of genes out of the whole genome of the selected organism. By applying on Bonferroni correction corrected p-value is used for pathway enrichment as a significance threshold. A correct p-value equal to 0.01 is used by this tool as a default threshold [110].

The pathways predicted by CTD are retrieved from KEGG PATHWAY and Reactome. KEGG PATHWAY (available at https://www.genome.jp/kegg/pathway.html) provides a vast collection of pathways related to metabolism, cellular processes, systems of organisms, processing of genetic knowledge, diseases and development of drugs. Pathway ID or gene names can be given as input as a result of which associated pathways are shown in result and clicking on a specific pathway figure and description [116].

The Reactome Knowledgebase (available at https://reactome.org) is a free open source pathway database and contains pathways related to cell cycle, signaling, neural functions, cell motility and immune system. In Reactome the pathways are supported by literature at every step. Biological processes are divided in to small molecular events and represented in Reactome. These events are grouped in order to form pathways. Input can be provided in the form of common gene name, gene ID, pathway ID, or pathway name. As a result of which pathway related to the search terms are displayed along with description of pathway [117].

3.6 Validation of Association of Metabolites with ASD

By following the above mentioned steps, the metabolites categorized as ASD associated is further validated by comparing them to reported metabolites in ASD patients. Literature search is performed on PubMed, Google Scholar, MEDLINE using keywords ASD, microbiota in ASD, gut metabolites and ASD, gut dysbiosis and ASD, disturbance of gut microbiome in ASD. List of gut metabolites that are associated with ASD patients are retrieved. This helps us to validate the metabolites predicted to be associated with ASD.

Chapter 4

Results and Discussion

In this chapter, the results of each step of the designed methodology are given in detail. The complete results along with the details given by tools and databases are discussed thoroughly. The intermediate steps details such as analysis of the output generated by the tools is also provided in this section. The results summary after performing each step is clearly shown in the form of tables and figures. The results of each objective are discussed under separate headings in order to avoid any confusion. At the end of this chapter, a summary of all results is given.

4.1 Extraction of Human Gut Metabolites Data

For investigating the relationship between gut microbiota and ASD onset and development, human gut metabolites data was retrieved from HMDB 4.0 (Human Metabolome Database) that is the most comprehensive human metabolome database up to date (available at http://www.hmdb.ca/). It consists of 114,160 small molecule human metabolites, out of which metabolites of microbial origin were selected. By applying this filter, a list of 172 human gut metabolites of microbial origin is obtained. The result consisted of name, HMDB ID, smiles structure, formula, average mass, monoisotopic mass and bio specimen location of metabolites.

4.2 Identification of Human Gut Metabolite-Protein Interactions

In order to reveal the role of identified human gut metabolites, proteins that show interaction with these metabolites were searched. For this purpose, STITCH 5 (Search Tool for Interactions of Chemicals) was used (available at http://stitch.embl.de/) because it is a large database that encompasses interaction data between 500,000 molecules and 9.6 million proteins. Search has been performed on STITCH 5 by inputting metabolite name and selecting Homo sapiens from organism list. For two metabolites out of 172 metabolites, smiles structure was used to predict interacting proteins.

For each metabolite result consisted of name of protein that showed interaction with metabolite along with three types of interaction scores that were "experimentally determined interaction score", database annotated score, "automated text mining interaction score" and combined score.

Combined score ranges from 0 to 1 where 0 means "no interaction", "0.4" means "medium confidence" and "0.7" is the default cut off value for highly confident interactions. For 35 metabolites, no protein showed interaction. For further steps proteins that showed high confidence interaction score (>=0.7) were selected for each metabolite.

4.3 Analysis of Connections between Metabolites and ASD at Genetic Level

In this step it had been evaluated that if there is any connection between gut microbial metabolites and ASD at genetic level. This has been done by checking the genes interacting with metabolite were also reported for ASD. A metabolite that is interacting with genes that are involved in ASD is considered to be important in development of ASD.

4.3.1 Extraction of ASD Genes

For getting ASD associated genes, the most comprehensive database of ASD, Autism KnowledgeBase 2.0 (Autism KB 2.0) with 3075 genes, out of which 171 genes are designated as core data set because of having high confidence score. These 171 genes consist of 99 syndromic and 109 non-syndromic autism genes. This core data set of genes was retrieved for analysis.

4.3.2 Prioritization of Gut Microbial Metabolites on the Basis of Shared Genes with ASD

To check if any genetic connection exists between gut microbial metabolites and ASD, for each of 172 metabolites search has been performed for finding common genes between metabolite interacting genes and ASD related genes. As a result of this search, 13 metabolites shared genes with ASD related gene set. Out of these thirteen metabolites, two metabolites shared two genes with ASD gene set while all others shared a single gene with ASD. The metabolites that showed genes in common with ASD along with number of genes and gene symbols are shown in table 4.1.

Table 4.1: Gut microbial metabolites that showed genetic connections with ASD

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	High confident interacting proteins (STITCH 5 s core >0.7)	Number of shared genes with ASD genes data set	Shared Genes
1.	Dimethylamine	HMDB0000087	4	4	1	MAOA
2.	Phenylacetic acid	HMDB0000209	11	9	1	PAH
3.	Succinic acid	HMDB0000254	17	14	1	RELN
4.	Indoxyl sulfate	HMDB0000682	21	15	1	CADM1
5.	4-Hydroxy phenylpyruvic acid	HMDB0000707	37	34	1	PAH
6.	4-Hydroxy butyric acid	HMDB0000710	9	4	2	GABRB1, ALDH5A1
7.	Tartaric acid	HMDB0000956	11	10	2	PTEN, COMT
8.	Trehalose	HMDB0000975	10	10	1	UPP2

Table 4.1 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	High confident interacting proteins (STITCH 5 s core >0.7)	Number of shared genes with ASD genes data set	Shared Genes
9.	3,4-Dihydroxy benzeneacetic acid	HMDB0001336	21	18	1	COMT
10.	N-Acetyl putrescine	HMDB0002064	12	10	1	MAOA
11.	Cadaverine	HMDB0002322	15	9	1	GAD1
12.	Methane	HMDB0002714	12	3	1	UPP2
13.	Phenylet hylamine	HMDB0012275	25	22	1	MAOA

4.4 Analysis of Connections between Gut Metabolites and ASD at Functional Level

Connections between gut metabolites and ASD have been analyzed by using two approaches. In first approach association of gut microbial metabolites with brain was checked. The other approach was based on functional enrichment analysis and pathway analysis of metabolites.

4.4.1 Assessment of Association of Human Gut Metabolites with Brain

For the assessment of association of human gut metabolites with brain is performed by testing two properties, one was involvement of metabolite in any psychological disorder and the other was the ability of metabolite to cross blood brain barrier.

4.4.1.1 Involvement of Metabolites in Mental Disorders

For finding the metabolites that was involved in mental disorders, inference of diseases has been done for all gut microbial metabolites. This inference of diseases has been performed by using Set Analyzer a tool of Comparative Toxicogenomics Database (CTD). In this tool, enrichment analysis has been performed for each metabolite by using interacting genes as input data set and selecting p-value 0.01 that is the default p-value for enrichment analysis.

As a result of this output appeared in the form of disease, disease category, p-value, corrected p-value, number of annotated genes and genome frequency. Diseases were filtered by selecting mental and nervous system disorders as disease category. By this analysis it has been found that 30 out of 172 metabolites were involved in some nervous system and mental disorders. Out of these 30, 19 metabolites were found to be associated with disorders that are ASD related as per literature.

4.4.1.2 Prediction of BBB Penetration Properties of Metabolites

In order to determine the possible effects of metabolites on brain function, the blood brain barrier crossing ability of metabolites was analyzed. This blood brain barrier crossing ability of metabolites was predicted by using webserver admetSAR2.0, that utilizes "quantitative structure activity relationship (QSAR) models"" to analyze properties such as absorption and distribution of chemicals.

This server was selected because it contains over 200,000 ADMET data points for 96000 unique compounds and it also provides a user-friendly interface. Smiles structures of metabolites were used to predict BBB permeability for metabolites. The result of admetSAR 2.0 consists of forty seven models that are intestinal absorption, blood–brain barrier penetration, hERG inhibitors, and many others[114]. This analysis was performed for all 172 metabolites and 138 metabolites were predicted to cross BBB.

4.4.1.3 Filtration of Brain Associated Human Gut Microbial Metabolites

After performing both aforementioned analyses metabolites that were found to be associated with any mental or nervous system disorder related to ASD and were also predicted to cross BBB were said to brain associated human gut microbial metabolites. Only 18 out of 172 metabolites were found to have both properties, these were considered for further analysis. The details of these metabolites are given in table 4.2.

TABLE 4.2: List of brain associated human gut microbial metabolites

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
1.	Butyric acid	HMDB0000039	19	+	Sleep Disorders, Spasm, Neuromuscular Manifestations, Sleep Disorders, Hyperkinesis
2.	Acetic acid	HMDB0000042	13	+	Alzheimer Disease, Dementia, Neurocognitive Disorders, Learning Disorders, Tauopathies, Cerebrovascular disorders

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
3.	Dimethylamine	HMDB0000087	4	+	Huntington Disease, Cognition Disorders, Dementia, Hepatic Encephalopathy Chorea, Parkinson Disease, Parkinsonian Disorders
4.	Dimethylglycine	HMDB0000092	11	+	Hepatolenticular Degeneration
5.	Phenylpyruvic acid	HMDB0000205	27	+	Tyrosinemias

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
6.	Propionic acid	HMDB0000237	31	+	"Attention Deficit Disorder with Hyperactivity", "Attention Deficit and Disruptive Behavior Disorders" , Neurologic Manifestations Epilepsy, Temporal Lobe Epilepsies
7.	Pyruvic acid	HMDB0000243	30	+	Pyruvate Dehydrogenase Complex Deficiency Disease, Brain Diseases, Inborn Brain Diseases,

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
8.	Indoxyl sulfate	HMDB0000682	15	+	Depressive Disorder, Mood Disorders, Meningitis, Aseptic Stroke, Brain Ischemia, Meningitis, Cerebrovascular Disorders

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
9.	Mannitol	HMDB0000765	20	+	"Mental Disorders", "Alcohol-Related Disorders", "Neurodevelopmental Disorders", Status Epilepticus, Seizures, Diabetic Neuropathies, "Cerebrovascular Disorders", "Movement Disorders", Stroke Trauma, Brain Ischemia, Basal Ganglia Diseases

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
10.	Isopropyl alcohol	HMDB0000863	20	+	Mental Disorders, "Brain Ischemia", "Cerebrovascular Disorders", "Central Nervous System Diseases", "Neurotoxicity Syndromes", "Heavy Metal Poisoning"
11.	Taurodeoxycholic acid	HMDB0000896	8	+	Peripheral Nervous System Diseases
12.	Tartaric acid	HMDB0000956	10	+	Autistic Disorder
13.	Hydrogen	HMDB0001362	14	+	Basal Ganglia Diseases, Spinal Cord Diseases, Movement Disorders

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
14.	p-Cresol	HMDB0001858	13	+	Alzheimer Disease, Dementia, Neurocognitive Disorders, Seizures Cerebrovascular Disorders, Movement Disorders, Status Epilepticus, Alzheimer Disease, Tauopathies Tremor Neurotoxicity Syndromes, Neurologic Manifestations Dementia, Neurodegenerative Diseases, Basal Ganglia Diseases, Cerebral Hemorrhage

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
15.	N-Acetylputrescine	HMDB0002064	10	+	Huntington Disease, Hepatic Encephalopathy, Pheochromocytoma, Chorea Paraganglioma, Liver Failure, Hepatic Insufficiency Hypotension, Cognition Disorders, Parkinson ,Disease, Huntington Disease, Hepatic Encephalopathy, Chorea, Parkinson Disease
16.	Methane	HMDB0002714	3	+	Mental Disorders

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
17.	Phenylethylamine	HMDB0012275	22	+	Huntington Disease, Mental Disorders, Schizophrenia Spectrum, Sleep Wake Disorders, Movement Disorders, Parkinson Disease, Parkinsonian Disorders, Huntington Disease, Hepatic Encephalopathy, Chorea, Basal Ganglia Diseases, Dyskinesias, Sleep Wake Disorders

Table 4.2 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	BBB crossing ability	Associated Diseases
18.	Piperidine	HMDB0034301	5	+	"Cocaine-Related Disorders", "Substance-Related Disorders", "Opioid-Related Disorders Mental Disorders", Hyperalgesia

4.5 Functional Analysis of Metabolites

Brain associated gut metabolites were further analyzed by two ways, firstly functional enrichment analysis was performed after that pathway analysis was performed in order to find out the mechanism underlying the involvement of these metabolites in autism spectrum disorder.

4.5.1 Functional Enrichment Analysis

Functional enrichment analysis provides the functional annotation of a set of compounds, so it gives insights into the overrepresented functions of the compounds. These years functional enrichment analysis is frequently used in the field of metabolomics for the analysis of metabolites. This analysis was performed on the brain associated gut metabolites by using two tools one is Set analyzer of Comparative Toxicogenomics Database.

4.5.1.1 Enrichment Analysis of Gene Ontology of Brain Associated Human Gut Metabolites

For performing enrichment analysis, a tool of Comparative Toxicogenomics Database, has been used. It provided the gene ontology terms that are enriched for metabolites, where gene ontology (GO) describe functions of protein in three different categories named as cellular component (CC), molecular function (MF) and biological process (BP) [111]. CTD is a powerful database that was used for analysis of metabolites using proteins that showed interaction with each metabolite. A significance threshold that is p value was set as 0.01 that is the default p value of the tool. This analysis was first applied on the set of ASD genes retrieved from Autism KB. It gave 903 gene ontology terms as an output for the set of ASD genes. The result included gene ontology type, GO term, p-value, annotated genes number, symbols of annotated genes and genome frequency. After this the same analysis was performed for each of 18 brain associated gut metabolites. The

resultant GO terms for all metabolites were analyzed to find out how many GO terms are shared for each metabolite with ASD data set GO terms. It was found that out of 18 brain-associated gut metabolites, one metabolite that is methane did not shared any GO term with ASD GO terms so it was excluded from list of metabolites for further analysis. The details of metabolites that shared GO terms with ASD GO terms are shown in table 4.3.

TABLE 4.3: List of metabolites that shared GO terms with ASD gene set

Sr. No.	Metabolite name	Metabolite HMDB ID	Total intera-cting proteins (STITCH 5)	Number of total Go terms	Number of shared GO terms with ASD
1.	Butyric acid	HMDB0000039	19	15	14
2.	Acetic acid	HMDB0000042	13	23	9
3.	Dimethy lamine	HMDB0000087	4	42	10
4.	Dimethyl glycine	HMDB0000092	11	32	10
5.	Phenyl pyruvic acid	HMDB0000205	27	56	6
6.	Propionic acid	HMDB0000237	31	54	34
7.	Pyruvic acid	HMDB0000243	30	72	14
8.	Indoxyl sulfate	HMDB0000682	15	44	25
9.	Mannitol	HMDB0000765	20	129	73

Table 4.3 continued from previous page

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	Number of total Go terms	Number of shared GO terms with ASD
10.	Isopropyl alcohol	HMDB0000863	20	84	38
11.	Taurodeoxy cholicacid	HMDB0000896	8	162	90
12.	Tartaric acid	HMDB0000956	10	30	16
13.	Hydrogen	HMDB0001362	14	28	11
14.	p-Cresol	HMDB0001858	13	84	48
15.	N-Acetyl putrescine	HMDB0002064	10	32	10
16.	Phenyl ethylamine	HMDB0012275	22	38	16
17.	Piperidine	HMDB0034301	5	14	2

4.5.2 Pathway Analysis of Metabolites

In order to analyze the biological context of metabolites, set analyzer of comparative toxicogenomics database, KEGG PATHWAY and Reactome Knowledgebase. First of all this was performed using set analyzer of CTD by using significance level that is p value at 0.01. This analysis was first applied on the set of ASD genes retrieved from Autism KB. It gave 176 pathways as an output for the set of ASD genes. The result included pathway name, pathway ID, p-value, corrected p-value, number of annotated genes, symbols of annotated genes and genome frequency. After this the same analysis was performed for each of 16 brain associated

gut metabolites. The resultant pathways for all metabolites were analyzed to find out how many pathways were shared for each metabolite with pathways of ASD data set. It was found that out of 16 brain-associated gut metabolites, seven metabolites shared one or more pathways with ASD associated pathways. These pathways were further studied on KEGG Pathway and Reactome Knowledgebase. The summary of pathway sharing for metabolites is given in table 4.4.

Table 4.4: Summary of pathway sharing for metabolites

Sr. No.	Metabolite name	Metabolite HMDB ID	Total interacting proteins (STITCH 5)	Total Pathways (Set Analyzer: CTD)	Number of shared pathways with ASD pathways set	Pathway details	
						Pathway ID	Pathway name
1.	Acetic acid	HMDB0000042	13	6	2	REACT:R-HSA-112311	Neurotransmitter Clearance In The Synaptic Cleft
						REACT:R-HSA-112315	Transmission across Chemical Synapses

Table 4.4 continued from previous page

2.	Dimethylamine	HMDB0000087	4	21	3	KEGG:hsa04726	Serotonergic synapse
						KEGG:hsa04728	Dopaminergic synapse
						KEGG:hsa05034	Alcoholism
3.	Mannitol	HMDB0000765	20	18	4	REACT:R-HSA-168256	Immune System
						REACT:R-HSA-168249	Innate Immune System
						KEGG:hsa05205	Proteoglycans in cancer
4.	Isopropyl alcohol	HMDB0000863	20	20	5	KEGG:hsa04071	Sphingolipid signaling pathway
						KEGG:hsa04722	Neurotrophin signaling pathway
						KEGG:hsa04210	Apoptosis

Table 4.4 continued from previous page

						KEGG:hsa04921	Oxytocin signaling pathway
						KEGG:hsa04010	MAPK signaling pathway
5.	p-Cresol	HMDB0001858	13	28	2	REACT:R-HSA-166016	Toll Like Receptor 4 (TLR4) Cascade
						KEGG: hsa04210	Apoptosis
6.	N-Acetylputrescine	HMDB0002064	10	19	3	KEGG: hsa04726	Serotonergic synapse
						KEGG: hsa04728	Dopaminergic synapse
						KEGG: hsa05034	Alcoholism

Table 4.4 continued from previous page

7.	Phenylethylamine	HMDB0012275	22	30	4	KEGG: hsa05034	Alcoholism
						KEGG: hsa04080	Neuroactive ligand-receptor interaction
						KEGG: hsa04726	Serotonergic synapse
						KEGG :hsa04728	Dopaminergic synapse

4.5.2.1 ASD Associated Pathways for Acetic Acid

The pathways found by set analyzer of CTD for the genes associated with acetic acid, consisted of two pathways that were shared with the pathways associated with ASD gene set. These two pathways were neurotransmitter clearance in the synaptic cleft and transmission across synapse. These two were retrieved from Reactome Knowledgebase. In the neurotransmitter clearance pathway, when neurotransmitters are released in the synaptic cleft, then the extra quantity of neurotransmitters is removed so that it does not cause extra stimulation of the post synaptic neuron. Neurotransmitter can be performed by degrading enzymes, astrocytes or presynaptic axon. It has been reported that the dysregulation of synaptic functions including changes in neurotransmission leads to defects in nervous system and act as one of the molecular basis of ASD [118]. This pathway is shown in following figure. The extra quantity of neurotransmitters is removed so that it

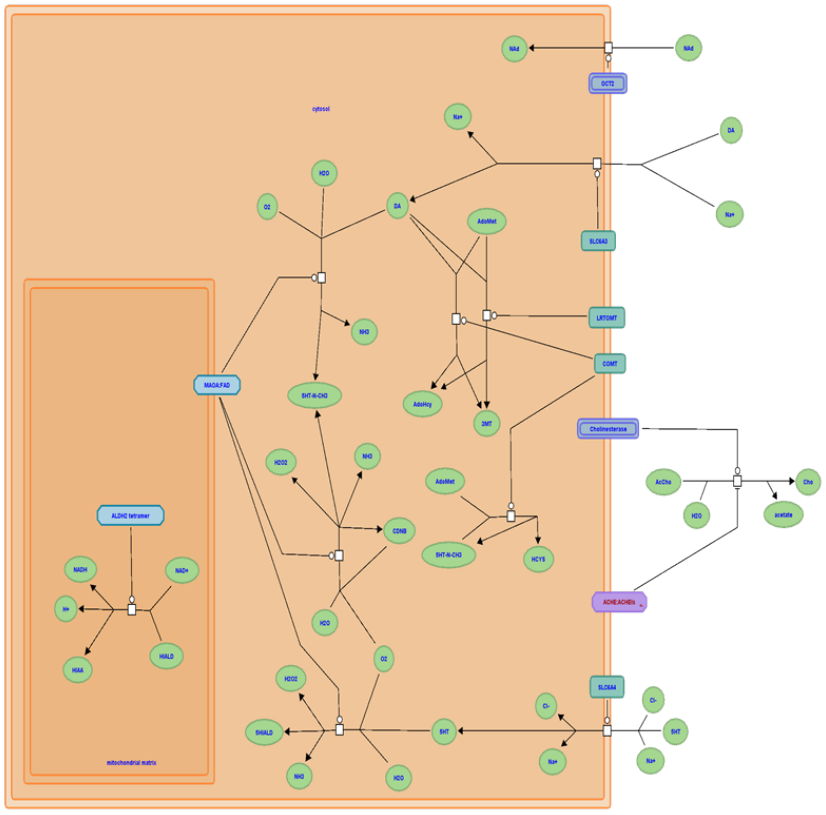

FIGURE 4.1: Pathway of Neurotransmitter Clearance In The Synaptic Cleft[117]

does not cause extra stimulation of the post synaptic neuron in neurotransmitter clearance pathway.

The second pathway associated with acetic acid is the transmission across chemical synapses. The communication between neurons occurs in the form of synapse. This communication involves the release of chemicals that are known as neurotransmitters. The disturbance of this transmission of signals from one neuron to another can lead to dysregulation of neural functions and it can result in affected behaviors associated with ASD [119],[120]. This is depicted in following figure 4.2.

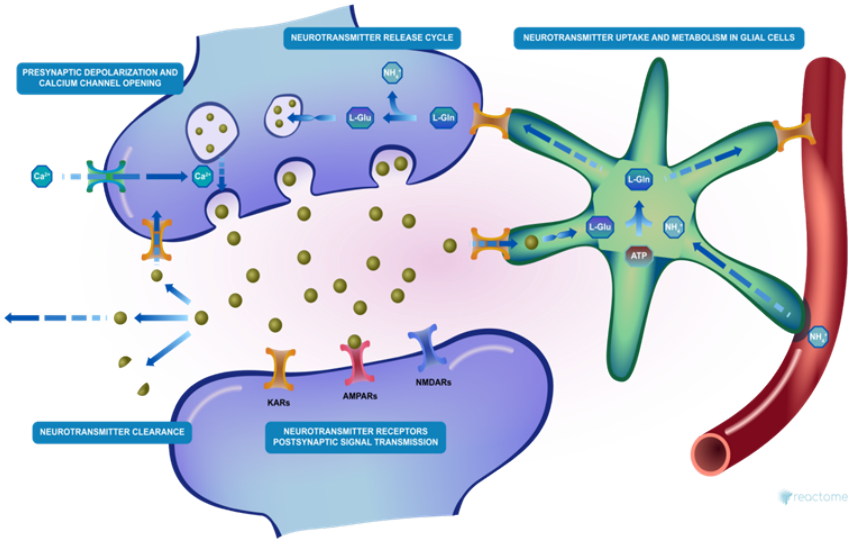

FIGURE 4.2: Pathway of transmission across chemical synapses by which the communication occurs between neurons [117].

4.5.2.2 ASD Associated Pathways for Dimethylamine

The pathways found by set analyzer of CTD for the genes associated with dimethylamine, consist of three pathways that are shared with the pathways associated with ASD gene set. These pathways are serotonergic synapse, dopaminergic synapse and alcoholism. These pathways were retrieved from KEGG PATHWAY. Serotonin is a neurotransmitter that is involved in the process of learning, emotions, memory and sleep. It has been reported that high levels of serotonin are observed in greater than one fourth of the ASD individuals. This is also used as a biological marker for this disorder [121]. This pathway is shown in figure 4.3.

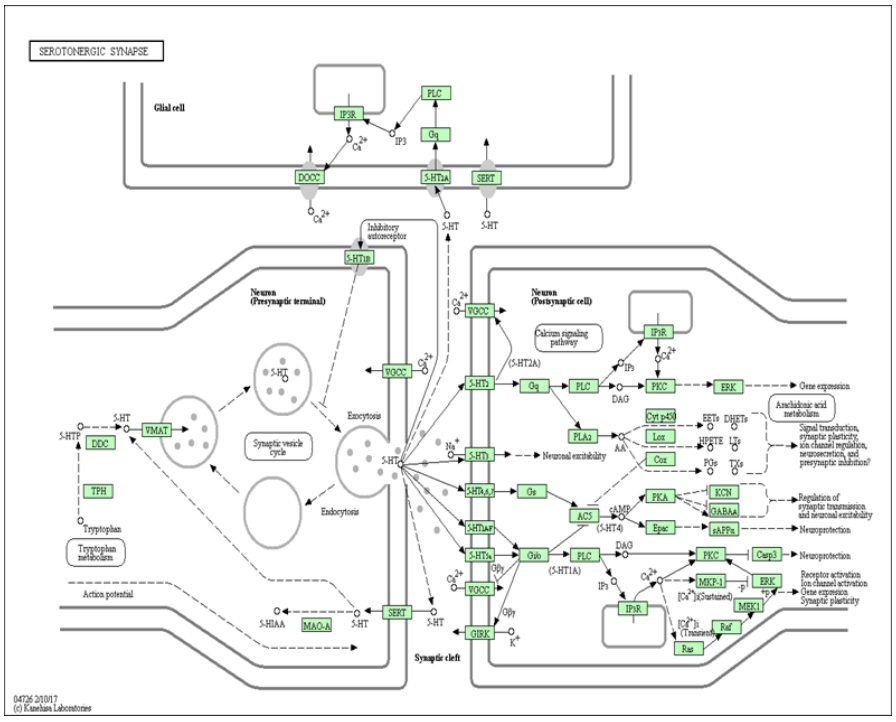

FIGURE 4.3: Pathway of serotonergic synapse [116] This pathway is affected in ASD because high levels of serotonin are observed in greater than one fourth of the ASD individuals [121]

The second pathway for dimethylamine is dopaminergic synapse. Dopamine is a neurotransmitter that is involved in functions such as learning, reward and memory. Dopamine acts through five types of receptors in nervous system. The dysregulation of dopamine signaling can lead to social deficits and occurrence of limited behaviors in ASD subjects. The group of neurons associated with dopamine has great effects on activities related to behaviors observed in ASD[122][123]. This is shown in figure 4.4.

Alcoholism is the third pathway found to be associated with dimethylamine. Alcoholism is a chronic disorder and it has serious negative effects on health. In alcoholism, dopamine release is affected and PKA signaling is also changed. Alcoholism is reported to be associated with ASD in many studies but the underlying mechanism of its involvement in ASD is not clearly understood yet [124]. The alcoholism pathway is shown in figure 4.5.

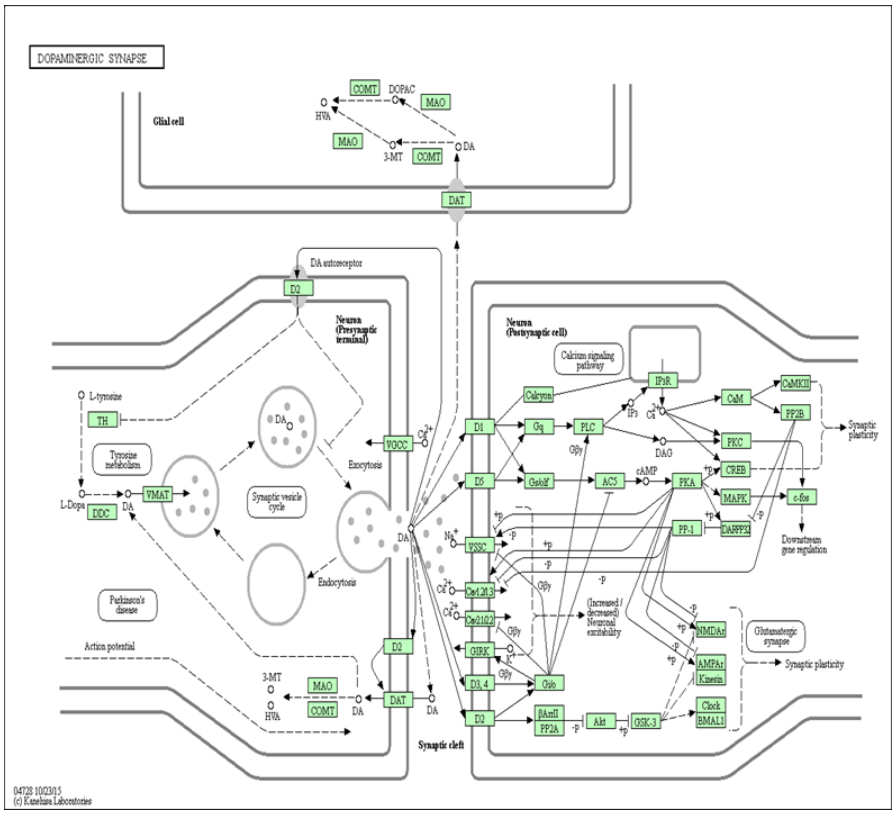

FIGURE 4.4: Pathway of dopaminergic synapse [116] The dysregulation of dopamine signaling can lead to social deficits and occurrence of limited behaviors in ASD subjects [122].

4.5.2.3 ASD Associated Pathways for Mannitol

The pathways found by set analyzer of CTD for the genes associated with mannitol, consist of three pathways that were also found in the pathways associated with ASD gene set. These pathways are immune system, innate immune system and proteoglycans in cancer. The immune system and innate immune system pathway were retrieved from Reactome whereas "Proteoglycans in cancer" was retrieved from KEGG PATHWAY.

Our immune system is designed to avoid infection in our bodies when we are exposed to any foreign agent by food, inhalation etc. The role of immune system in ASD is very important because mediators of immune system are involved in affecting many central nervous system functions. Some cytokines, T cells and natural killer cells have shown disturbed activity such as some cytokines can cause

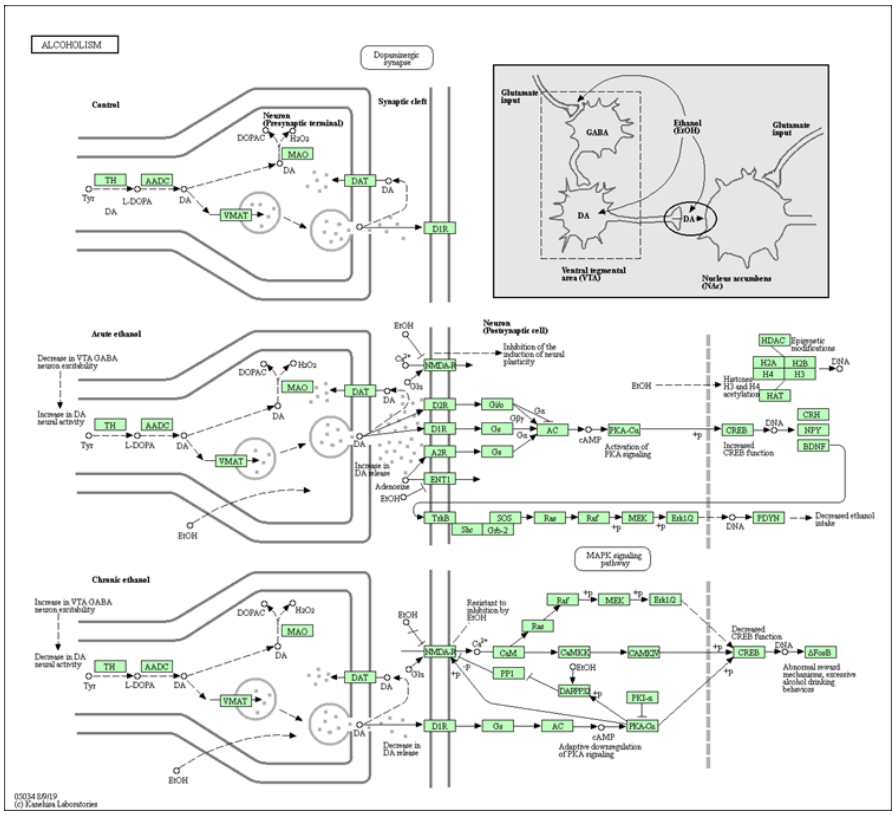

FIGURE 4.5: Pathway of alcoholism [116] Alcoholism is a chronic disorder and it has serious negative effects on health. In alcoholism, dopamine release is affected and PKA signaling is also changed [124].

neuron cells death. These changes can affect neural functions and cause ASD like behaviors [125]. This is shown in figure 4.6.

Innate immune system was also found to be associated with mannitol. The innate immune system comprises of the natural immunity present in an individual by birth. The role of immune system in ASD is very important because mediators of immune system are involved in affecting many central nervous system functions. Some cytokines, T cells and natural killer cells have shown disturbed activity such as some cytokines can cause neuron cells death. These changes can affect neural functions and cause ASD like behaviors [125]. This is shown in figure 4.7.

The third pathway associated with dimethylamine is proteoglycans in cancer. These proteoglycans are of four main categories and are involved in proliferation of tumor cells and some are also involved in tumor growth repression. By

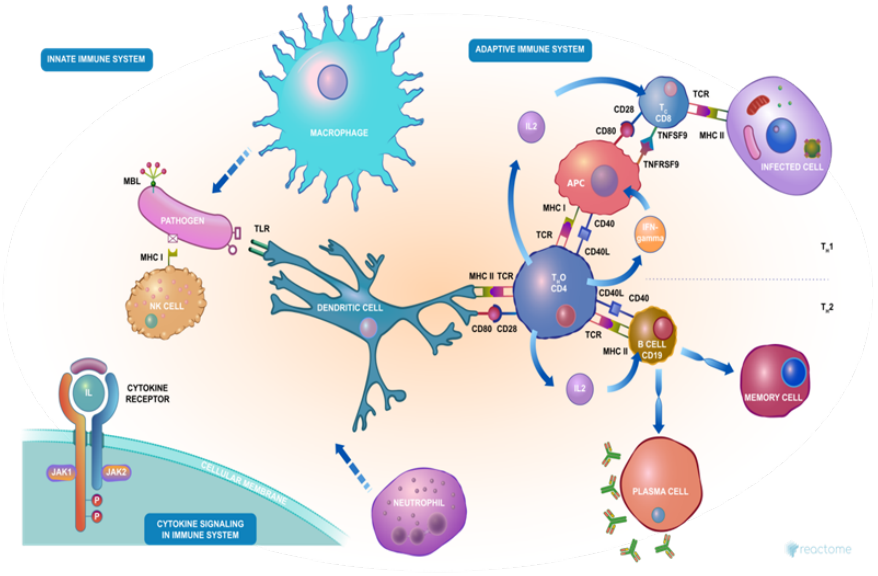

FIGURE 4.6: Immune system pathway; our immune is system is designed to avoid infection in our bodies when we are exposed to any foreign agent by food, inhalation [117].

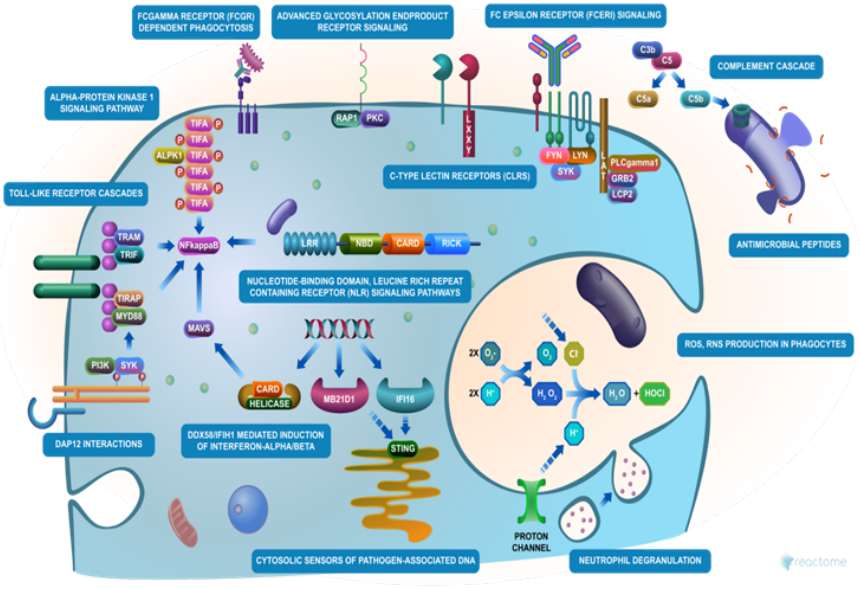

FIGURE 4.7: Innate immune system pathway; the innate immune system comprises of the natural immunity present in an individual by birth [117]

examining the brains of ASD patients, it has been found that there are some abnormalities of structure observed. The decrease in amount of heparin sulfate that is a proteoglycan can be involved in causing these abnormalities in ASD [126]. This pathway is shown in figure 4.8.

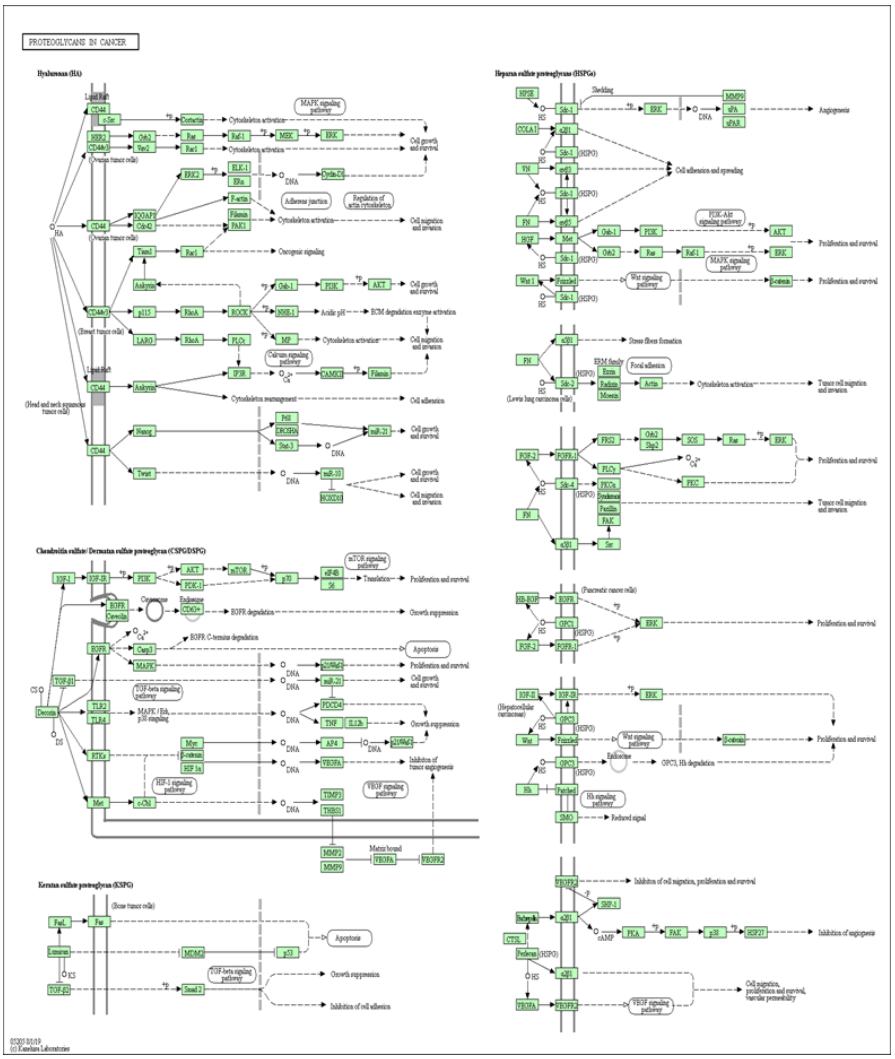

FIGURE 4.8: Pathway of proteoglycans in cancer; these proteoglycans are involved in proliferation of tumor cells and some are also involved in tumor growth repression [116]

4.5.2.4 ASD Associated Pathways for Isopropyl Alcohol

Five pathways were found by set analyzer of CTD for the genes associated with the metabolite, isopropyl alcohol, that were also found in the pathways associated with ASD gene set. These pathways were sphingolipid signaling pathway, neurotrophin signaling pathway, apoptosis, oxytocin signaling pathway and MAPK signaling pathway. These pathways were retrieved from KEGG PATHWAY.

Sphingomyelin is involved in many signaling pathways, and its products are bioactive compound such as ceramide. These products are involved in apoptosis pathway. The disturbance in sphingolipid molecules is involved in altered white matter

of cerebrum in autistic children [127]. This pathway is shown in figure 4.7.

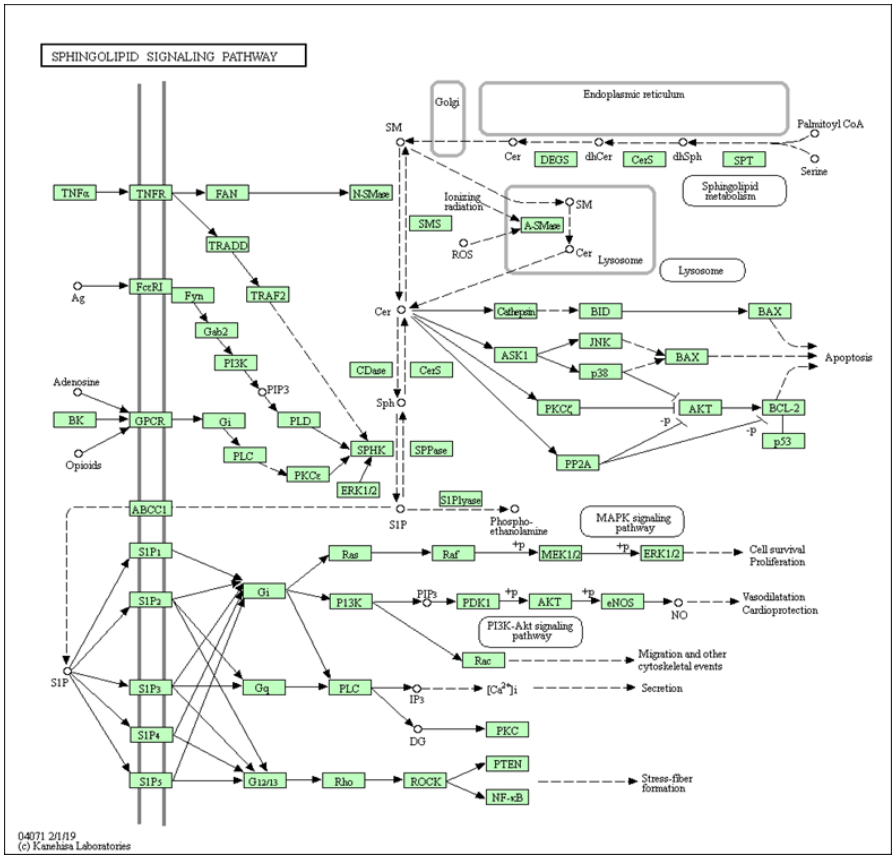

FIGURE 4.9: Sphingolipid signaling pathway; its products are involved in apoptosis pathway [116].

Isopropyl alcohol was also found to be associated with neurotrophin signaling pathway. Neurotrophins are the factors that play role in growth and differentiation of neurons. There are different types of these neurotrophins. These neurotrophins play role in pathways related to memory and learning. It is reported that the dysregulation of neurotrophins such as BDNF is involved in ASD. It has also shown in animal models that the autistic behaviors are linked with neurotrophin signaling [128]. This is shown by figure 4.10.

Apoptosis is also one of the pathways associated with iso propyl alcohol. Apoptosis is the process of removing the damaged cells by themselves. It is an important phenomenon because it clears the affected cells that can otherwise cause detrimental effects. It is has been shown by research that oxidative stress and apoptosis are highly involved in pathogenesis of ASD [129]. The process of apoptosis is shown in figure 4.11.

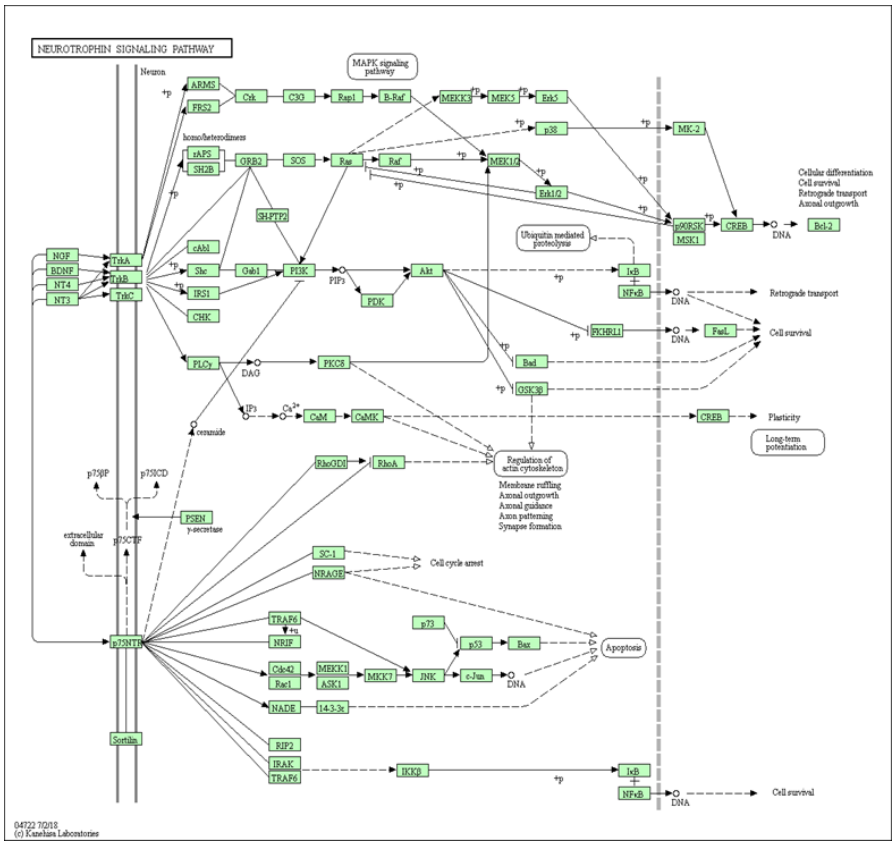

FIGURE 4.10: Neurotrophin signaling pathway; neurotrophins play role in growth and differentiation of neurons [116].

The fourth pathway found for isopropyl alcohol is oxytocin signaling pathway. Oxytocin is a neurotransmitter and it has many important functions of diverse types. Its functions that are related to ASD are its influence on social behavior. It can be used for therapy of ASD patients to improve symptoms of ASD related to cognition and social behavior [130]. This pathway is shown in figure 4.12.

The last pathway that is among the results for isopropyl alcohol is MAPK signaling pathway. MAPK pathway is involved in performing important functions such as growth and migration of cells. There are four types of these cells. It has been found that the MAPK genes are among the genes that have ASD related mutations. MAPK pathway plays important role in development of nervous system during fetal growth. Animal studies have also shown the dysregulation of this pathway in development of autism [131]. This is shown in figure 4.13.

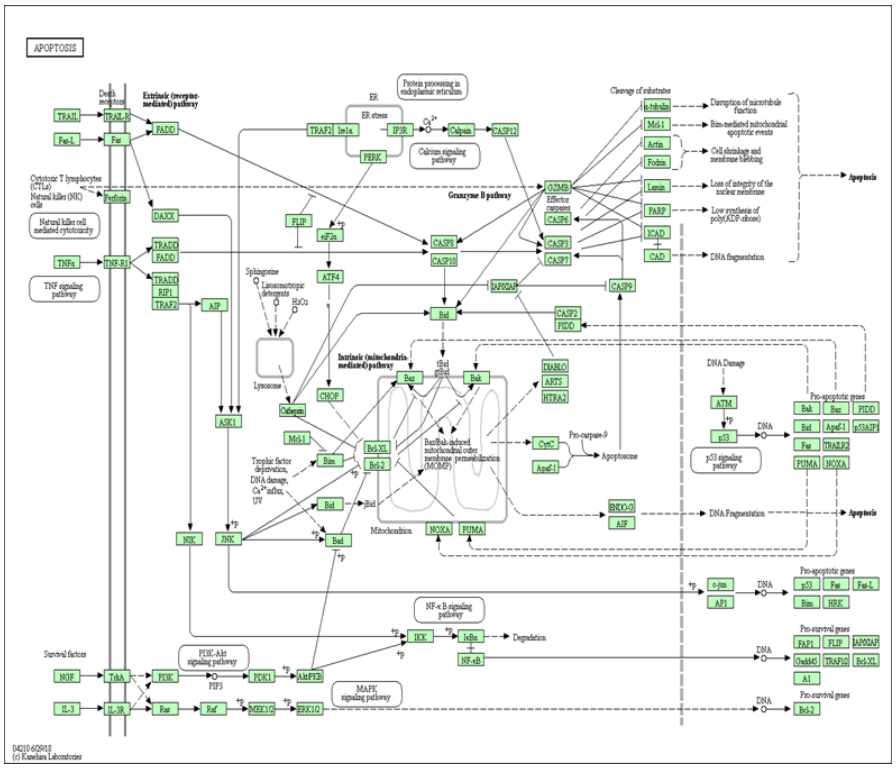

FIGURE 4.11: Pathway of Apoptosis involve process of removing the damaged cells by themselves it is also called programmed death [116].

4.5.2.5 ASD Associated Pathways for P-Cresol

The pathways found by set analyzer of CTD for the genes associated with p-cresol, consisted of two pathways that are shared with the pathways associated with ASD gene set. These pathways are "Toll like receptor 4 cascade" and apoptosis were retrieved from Reactome and KEGG PATHWAY respectively. Toll like receptor 4 cascade is one of the pathways found to be associated with p-cresol. TLR4 is a receptor involved in recognizing bacteria. TLR4 produces the mediators involved in inflammation such as IL6. It has been proved by literature that the pathogenesis of ASD involves the role of TLR4. It is found to be associated either increased inflammation and autistic behaviors in ASD individuals. The expression of these receptors is found to be increased in ASD individuals as compared to normal individuals. It has been suggested that if this TLR4 signaling is improved with drugs then the inflammation in ASD patients can be alleviated [132].

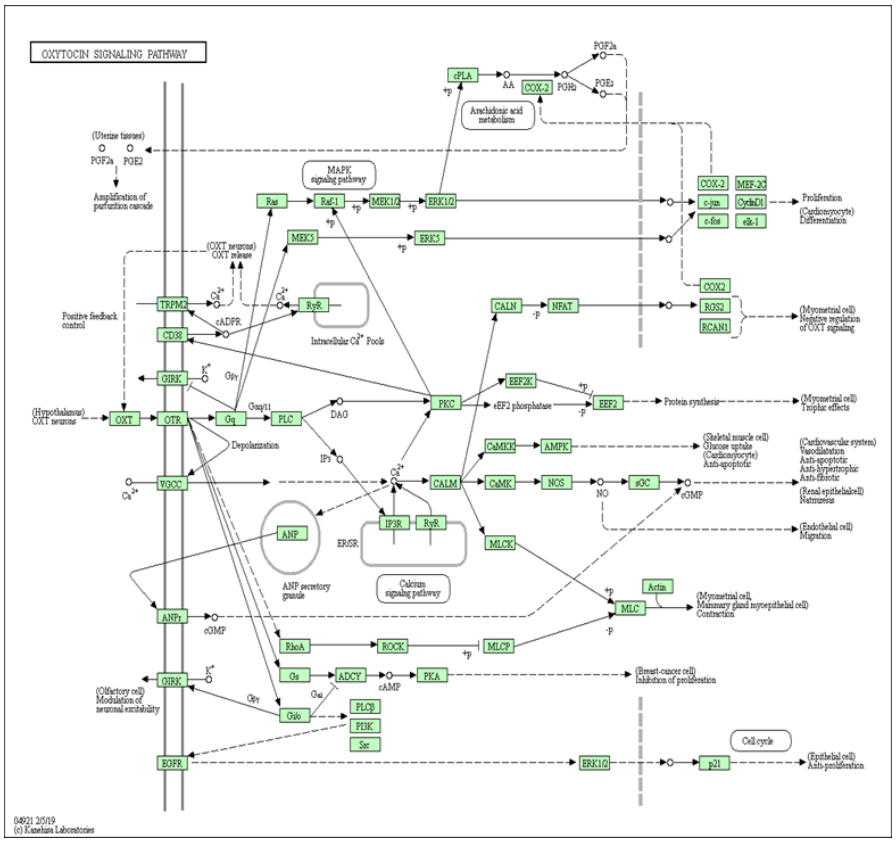

FIGURE 4.12: Oxytocin signaling pathway; oxytocin functions associated with ASD are its role in social behavior [116].

Apoptosis is also associated with p-cresol and it is already discussed earlier in pathways associated with isopropyl alcohol.

4.5.2.6 ASD Associated Pathways for N-Acetylputrescine

The pathways found by set analyzer of CTD for the genes associated with N-acetylputrescine, consist of three pathways that are shared with the pathways associated with ASD gene set. These pathways are serotonergic synapse, dopaminergic synapse and alcoholism. These pathways were also found to show association with dimethylamine and were discussed earlier.

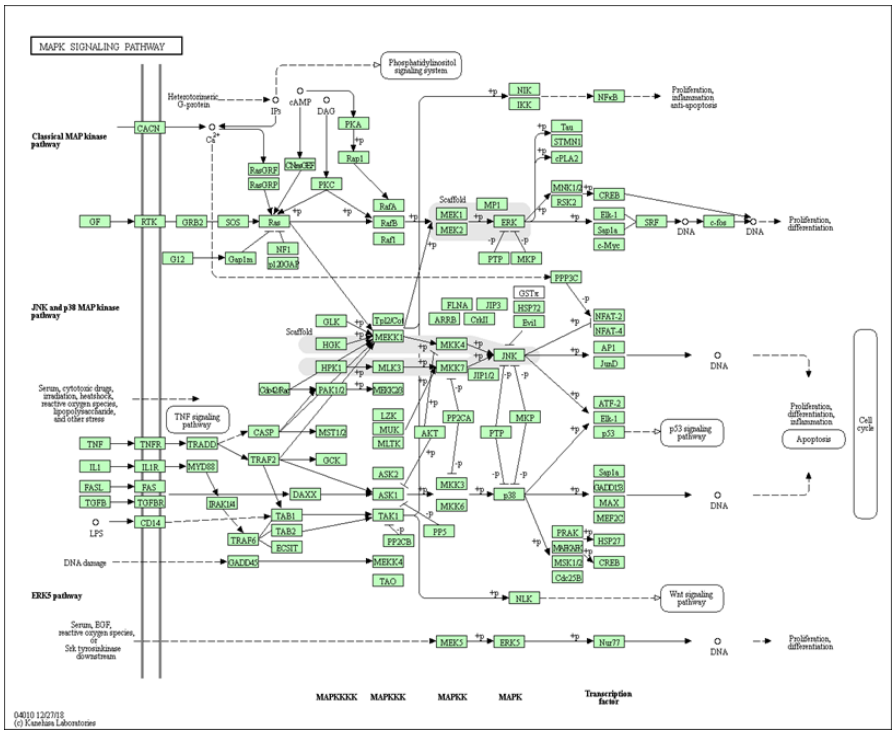

FIGURE 4.13: MAPK signaling pathway is involved in performing important functions such as growth and migration of cells [116]

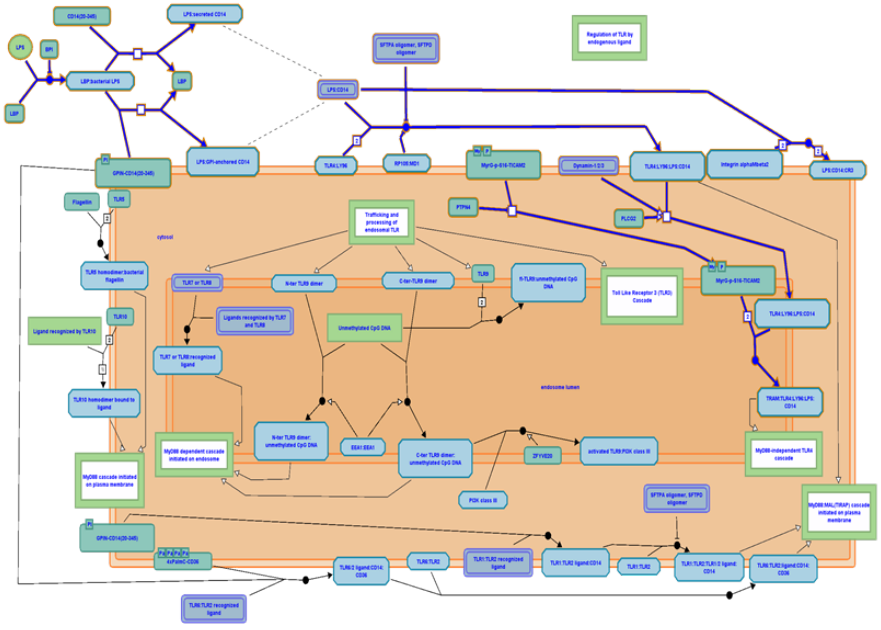

FIGURE 4.14: Toll like receptor 4 cascade; TLR4 is a receptor involved in recognizing bacteria. TLR4 produces the mediators involved in inflammation such as IL6. [117]

4.5.2.7 ASD Associated Pathways for Phenylethylamine

The pathways found by set analyzer of CTD for the genes associated with phenylethylamine, consist of four pathways that were shared with the pathways associated with ASD gene set. These pathways were serotonergic synapse, dopaminergic synapse, alcoholism, and neuroactive ligand receptor interaction. Three of these pathways were already discussed in pathways associated with dimethylamine whereas the neuroactive ligand receptor pathway was retrieved from KEGG PATHWAY. The interaction between the neuroactive compounds and receptors is of great importance in neural functions. This pathway is one of the top two significantly enriched signaling pathways associated with ASD performed in an analysis performed in a study [133]. It is also reported in a study that there is some down regulation of this pathway in ASD patients. Rikki Hullinger et al. found that neural activity dysregulation leads to symptoms of ASD [134].

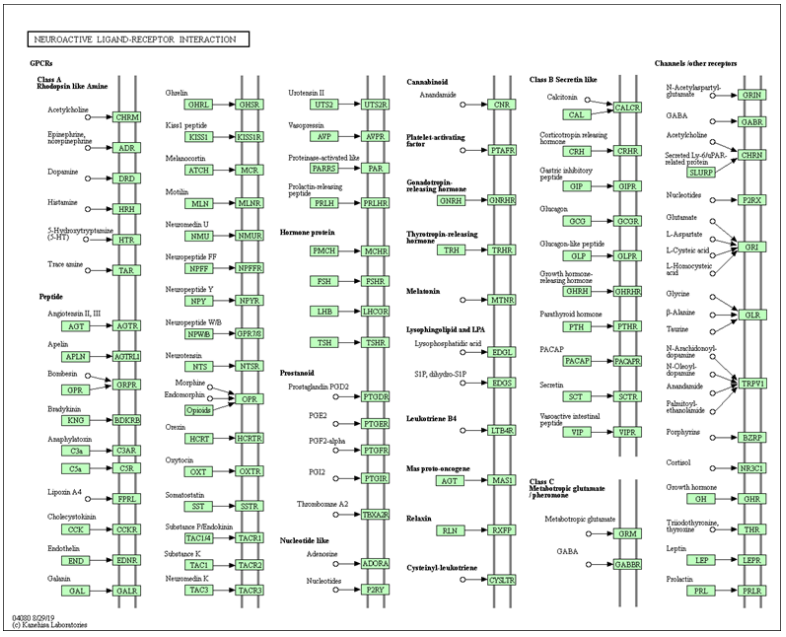

FIGURE 4.15: Pathway of neuroactive ligand-receptor interaction; the interaction between the neuroactive compounds and receptors is of great importance in neural functions [116]

4.6 Validation of Association of Metabolites with ASD

For the validation of the metabolites found by this study to have role in ASD by having some connections with this disorder, at genetic and functional level, literature search was performed. For this literature finding, search was performed in PubMed and Google scholar using key words like "ASD", "autism", "microbiota and ASD", "gut metabolites and ASD", "gut dysbiosis in ASD", "levels of metabolite in ASD" and names of metabolites. In this way the role of metabolite in ASD was investigated. According to our literature search 9 out of the 13 metabolites predicted to have genetic connections with ASD and 4 out of 7 metabolites predicted to have functional connections with ASD were validated. These results are shown in following table 4.5.

Table 4.5: Validation of associated metabolites from literature search

Sr. No.	Metabolite Name	HMDB ID	Association with ASD in literature	Role in pathogenesis of ASD	Change in metabolite level in ASD subjects	Ref
Metabolites having genetic connections with ASD						
1.	Dimethylamine	HMDB0000087	Found	Not discovered	Higher levels in urine samples	[135] [136] [137]
2.	Phenylacetic acid	HMDB0000209	Found	It changes metabolism of phenylalanine leading to ASD behavior	Higher levels in ASD subjects	[135]
3.	Succinic acid	HMDB0000254	Found	Not discovered	Higher levels in urine samples	[135]
4.	Indoxyl sulfate	HMDB0000682	Found	cognitive impairment	Higher levels in urine samples	[138]
5.	4-Hydroxy phenylpyruvic acid	HMDB0000707	Not found	-	-	-

Table 4.5 continued from previous page

6.	4-Hydroxybutyric acid	HMDB0000710	Not found	-	-	-
7.	Tartaric acid	HMDB0000956	Found	Not discovered yet	Higher levels in urine samples	[139]
8.	Trehalose	HMDB0000975	Not found	-	-	-
9.	3,4-Dihydroxy benzeneacetic acid	HMDB0001336	Found	Not discovered	Higher levels in ASD patients	[140]
10.	N-Acetylputrescine	HMDB0002064	Not found	-	-	-
11.	Cadaverine	HMDB0002322	Found	Not discovered	Higher levels in ASD patients	[141]
12.	Methane	HMDB0002714	Found	Associated with GI symptoms	Higher levels in ASD patients	[142]
13.	Phenylethylamine	HMDB0012275	Found	It acts as a modulator in the “nigrostriatal dopaminergic pathway” and also cause changes in noradrenergic function	Lower levels in urine samples	[143]

Table 4.5 continued from previous page

Metabolites having functional connections with ASD						
1.	Acetic acid	HMDB0000042	Found	Not discovered	Higher levels in fecal samples	[144]
2.	Dimethylamine	HMDB0000087	Found	Not discovered yet	Higher levels in urine samples	[135] [136] [137]
3.	Mannitol	HMDB0000765	Not found	-	-	-
4.	Isopropyl alcohol	HMDB0000863	Not found	-	-	-
5.	p-Cresol	HMDB0001858	Found	Increases severity of symptoms specially GI symptoms	Higher levels in urine samples	[145], [146]
6.	N-Acetylputrescine	HMDB0002064	Not found	-	-	-
7.	Phenylethylamine	HMDB0012275	Found	It acts as a modulator in the "nigrostriatal dopaminergic pathway" and also cause changes in noradrenergic function	Lower levels in urine samples	[143]

4.7 Summary of Results

This study analyzed the interaction between gut microbial metabolites and ASD at two levels that are genetic and functional level. Gut microbial metabolites retrieved from HMDB were 172 in number. For each of these gut metabolites, proteins that show interaction with them were searched in STITCH 5. The metabolites that shared genes with ASD were categorized as metabolites having genetic connections with ASD. For finding functional connections between gut microbial metabolites and ASD, it was evaluated that which metabolites have association with brain by checking involvement of metabolites in mental disorders and BBB crossing ability of metabolites. Functional analysis of brain associated metabolites was performed by using enrichment analysis of gene ontology and pathway analysis. At the end, literature search was performed in order to validate the gut microbial metabolites that were predicted to have genetic and functional connections with ASD. The summarized results are shown in figure 4.16.

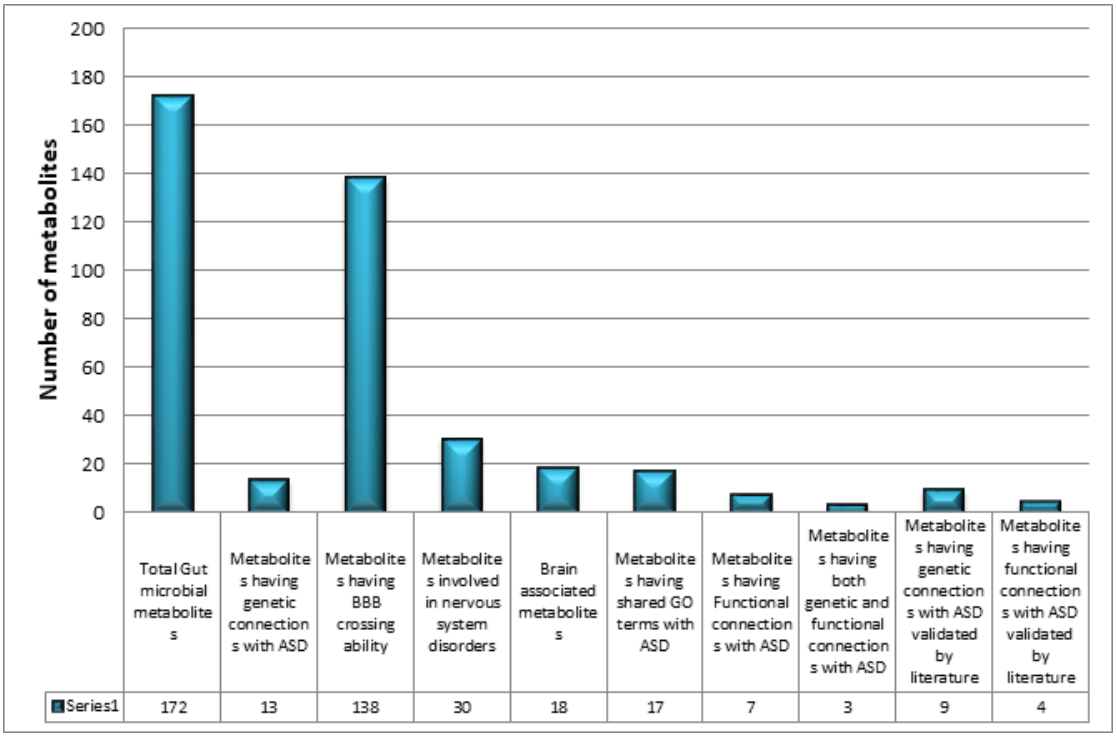

FIGURE 4.16: Graph representing number of metabolites belonging to different categories such as brain associated metabolites, metabolites having genetic connections with ASD and other categories

Chapter 5

Conclusions and Recommendations

This study is an effort to understand the mechanisms involved in etiology of Autism spectrum disorders. A computational methodology was designed by exploiting physico-chemical, genetic and functional data of gut microbial metabolites to figure out the role of microbiota in onset of ASD. Out of 172 gut microbial metabolites, 13 showed genetic connections with ASD. For evaluating functional connections the ability to cross BBB and involvement in nervous system disorders were checked, this gave 18 brain associated gut metabolites. Among the brain associated metabolites 7 shared gene ontology terms and pathways with ASD; these were designated as gut metabolites having functional connections with ASD. Literature survey proved that 9 out of the 13 metabolites having genetic connections 4 out of 7 metabolites having functional connections were also reported in ASD studies. These validated metabolites consist of acetic acid, dimethylamine, mannitol, isopropyl alcohol, p-cresol, n-acetylputrescine, phenylethylamine, phenylacetic acid, succinic acid, indoxyl sulfate, 4-hydroxyphenylpyruvic acid, 4-hydroxybutyric acid, tartaric acid, trehalose, 3,4-dihydroxybenzeneacetic acid, cadaverine, methane.

This study provided insight in to the role of microbiota in ASD development. This identification of ASD associated metabolites will open new doors for research on

treatment therapies for ASD as to date no targeted treatment is available for ASD. The metabolites identified can be evaluated experimentally in animal models to confirm their relation with ASD. This approach of narrowing down a large set of human gut metabolites can be replicated in any other disorder in which gut microbiota is acting as risk factor but its role is not discovered yet. Because of the in silico nature of this study, there is a large amount of knowledge generated by this study that can be utilized by other researchers in biomedical field to find out the mechanisms behind the role of gut microbiota in many other disorders.

Bibliography

[1] T. W. Robbins, "Cognition: The ultimate brain function," Neuropsychopharmacology, vol. 36, no. 1. pp. 1–2, Jan-2011.

[2] H. R. Park et al., "A short review on the current understanding of autism spectrum disorders," Experimental Neurobiology, vol. 25, no. 1. Korean Society for Neurodegenerative Disease, pp. 1–13, 01-Feb-2016.

[3] H. Faras, N. Al Ateeqi, and L. Tidmarsh, "Autism spectrum disorders," Ann. Saudi Med., vol. 30, no. 4, pp. 295–300, Jul. 2010.

[4] C. Yang et al., "AutismKB 2.0: A knowledgebase for the genetic evidence of autism spectrum disorder," Database, vol. 2018, no. 2018, Jan. 2018.

[5] J. Baio et al., "Prevalence of autism spectrum disorder among children aged 8 Years - Autism and developmental disabilities monitoring network, 11 Sites, United States, 2014," MMWR Surveill. Summ., vol. 67, no. 6, 2018.

[6] H. O. Salhia, L. A. Al-Nasser, L. S. Taher, A. M. Al-Khathaami, and A. A. El-Metwally, "Systemic review of the epidemiology of autism in Arab gulf countries," Neurosciences, vol. 19, no. 4, pp. 291–296, 2014.

[7] N. B. Almandil, D. N. Alkuroud, S. AbdulAzeez, A. AlSulaiman, A. Elaissari, and J. F. Borgio, "Environmental and Genetic Factors in Autism Spectrum Disorders: Special Emphasis on Data from Arabian Studies.," Int. J. Environ. Res. Public Health, vol. 16, no. 4, 2019.

[8] K. Lyall et al., "The Changing Epidemiology of Autism Spectrum Disorders," Annu. Rev. Public Health, vol. 38, no. 1, pp. 81–102, Mar. 2017.

[9] R. Raz et al., "Autism spectrum disorder and particulate matter air pollution before, during, and after pregnancy: a nested case-control analysis within the Nurses' Health Study II Cohort.," Environ. Health Perspect., vol. 123, no. 3, pp. 264–70, Mar. 2015.

[10] H. Ó. Atladóttir, T. B. Henriksen, D. E. Schendel, and E. T. Parner, "Autism after infection, febrile episodes, and antibiotic use during pregnancy: an exploratory study.," Pediatrics, vol. 130, no. 6, pp. e1447-54, Dec. 2012.

[11] H. K. Hughes, D. Rose, and P. Ashwood, "The Gut Microbiota and Dysbiosis in Autism Spectrum Disorders," Current Neurology and Neuroscience Reports, vol. 18, no. 11. Current Medicine Group LLC 1, 01-Nov-2018.

[12] C. Huttenhower et al., "Structure, function and diversity of the healthy human microbiome," Nature, vol. 486, no. 7402, pp. 207–214, Jun. 2012.

[13] J. M. Rodríguez et al., "The composition of the gut microbiota throughout life, with an emphasis on early life," Microb. Ecol. Heal. Dis., vol. 26, no. 0, Feb. 2015.

[14] M. van de Wouw, H. Schellekens, T. G. Dinan, and J. F. Cryan, "Microbiota-Gut-Brain Axis: Modulator of Host Metabolism and Appetite.," J. Nutr., vol. 147, no. 5, pp. 727–745, 2017.

[15] J. F. Cryan et al., Mind-altering microorganisms the impact of the gut microbiota on brain and behaviour of the nineteenth century through the pioneering work, Nat. Rev. Neurosci., vol. 13, no. 10, pp. 701-712, Oct. 2012.

[16] H. E. Vuong, J. M. Yano, T. C. Fung, and E. Y. Hsiao, "The Microbiome and Host Behavior," Annu. Rev. Neurosci., vol. 40, no. 1, pp. 21–49, Jul. 2017.

[17] E. Barrett, R. P. Ross, P. W. O'Toole, G. F. Fitzgerald, and C. Stanton, "γ-Aminobutyric acid production by culturable bacteria from the human intestine.," J. Appl. Microbiol., vol. 113, no. 2, pp. 411–7, Aug. 2012.

[18] V. Braniste et al., "The gut microbiota influences blood-brain barrier permeability in mice," Sci. Transl. Med., vol. 6, no. 263, Nov. 2014.

[19] C. J. Kelly et al., "Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function.," Cell Host Microbe, vol. 17, no. 5, pp. 662–71, May 2015.

[20] R. H. Sandler et al., "Short-term benefit from oral vancomycin treatment of regressive-onset autism.," J. Child Neurol., vol. 15, no. 7, pp. 429–35, Jul. 2000.

[21] J. Pulikkan, A. Mazumder, and T. Grace, "Role of the Gut Microbiome in Autism Spectrum Disorders," in Advances in Experimental Medicine and Biology, vol. 1118, Springer New York LLC, 2019, pp. 253–269.

[22] S. Carding, K. Verbeke, D. T. Vipond, B. M. Corfe, and L. J. Owen, "Dysbiosis of the gut microbiota in disease," Microb. Ecol. Heal. Dis., vol. 26, no. 0, Feb. 2015.

[23] L. W. Wang, D. J. Tancredi, and D. W. Thomas, "The prevalence of gastrointestinal problems in children across the United States with autism spectrum disorders from families with multiple affected members," J. Dev. Behav. Pediatr., vol. 32, no. 5, pp. 351–360, Jun. 2011.

[24] D. L. Coury et al., "Gastrointestinal conditions in children with autism spectrum disorder: Developing a research agenda," Pediatrics, vol. 130, no. SUPPL. 2. Nov-2012.

[25] B. O. McElhanon, C. McCracken, S. Karpen, and W. G. Sharp, "Gastrointestinal symptoms in autism spectrum disorder: A meta-analysis," Pediatrics, vol. 133, no. 5, pp. 872–883, 2014.

[26] T. Buie et al., "Recommendations for evaluation and treatment of common gastrointestinal problems in children with ASDs," Pediatrics, vol. 125, no. SUPPL. 1. Jan-2010.

[27] M. de Angelis, R. Francavilla, M. Piccolo, A. De Giacomo, and M. Gobbetti, "Autism spectrum disorders and intestinal microbiota," Gut Microbes, vol. 6, no. 3, pp. 207–213, Jan. 2015.

[28] J. Mead and P. Ashwood, "Evidence supporting an altered immune response in ASD," Immunology Letters, vol. 163, no. 1. Elsevier B.V., pp. 49–55, 01-Jan-2015.

[29] L. De Magistris et al., "Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives," J. Pediatr. Gastroenterol. Nutr., vol. 51, no. 4, pp. 418–424, Oct. 2010.

[30] X. Li et al., "Elevated immune response in the brain of autistic patients," J. Neuroimmunol., vol. 207, no. 1–2, pp. 111–116, Feb. 2009.

[31] P. Ashwood, P. Krakowiak, I. Hertz-Picciotto, R. Hansen, I. Pessah, and J. Van de Water, "Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome," Brain. Behav. Immun., vol. 25, no. 1, pp. 40–45, Jan. 2011.

[32] C. G. M. De Theije et al., "Pathways underlying the gut-to-brain connection in autism spectrum disorders as future targets for disease management," in European Journal of Pharmacology, 2011, vol. 668, no. SUPPL. 1.

[33] E. Y. Hsiao et al., "Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders," Cell, vol. 155, no. 7, pp. 1451–1463, Dec. 2013.

[34] M. De Angelis et al., "Fecal Microbiota and Metabolome of Children with Autism and Pervasive Developmental Disorder Not Otherwise Specified," PLoS One, vol. 8, no. 10, Oct. 2013.

[35] D. W. Kang et al., "Reduced Incidence of Prevotella and Other Fermenters in Intestinal Microflora of Autistic Children," PLoS One, vol. 8, no. 7, Jul. 2013.

[36] H. M. R. T. Parracho, M. O. Bingham, G. R. Gibson, and A. L. McCartney, "Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children," J. Med. Microbiol., vol. 54, no. 10, pp. 987–991, Oct. 2005.

[37] R. H. Sandler et al., "Short-term benefit from oral vancomycin treatment of regressive-onset autism," J. Child Neurol., vol. 15, no. 7, pp. 429–435, 2000.

[38] S. R. Sharma, X. Gonda, and F. I. Tarazi, "Autism Spectrum Disorder: Classification, diagnosis and therapy.," Pharmacol. Ther., vol. 190, pp. 91–104, 2018.

[39] N. Halfon and A. A. Kuo, "What DSM-5 could mean to children with autism and their families.," JAMA Pediatr., vol. 167, no. 7, pp. 608–13, Jul. 2013.

[40] E. J. Costello, S. Mustillo, A. Erkanli, G. Keeler, and A. Angold, "Prevalence and development of psychiatric disorders in childhood and adolescence," Arch. Gen. Psychiatry, vol. 60, no. 8, pp. 837–844, Aug. 2003.

[41] A. Minhas et al., "Parents' perspectives on care of children with autistic spectrum disorder in South Asia - Views from Pakistan and India," International Review of Psychiatry, vol. 27, no. 3. Taylor and Francis Ltd, pp. 247–256, 04-May-2015.

[42] E. U. Syed, S. A. Hussein, and S. Mahmud, "Screening for emotional and behavioural problems amongst 5-11-year-old school children in Karachi, Pakistan," Soc. Psychiatry Psychiatr. Epidemiol., vol. 42, no. 5, pp. 421–427, 2007.

[43] I. Mirza, A. Tareen, L. L. Davidson, and A. Rahman, "Community management of intellectual disabilities in Pakistan: a mixed methods study.," J. Intellect. Disabil. Res., vol. 53, no. 6, pp. 559–70, Jun. 2009.

[44] A. Sarwat, S. M. I. Ali, and M. S. Ejaz, "Mental health morbidity in children: A hospital based study in child psychiatry clinic," Pakistan J. Med. Sci., vol. 25, no. 6, pp. 982–985, 2009.

[45] N. Imran, M. R. Chaudry, M. W. Azeem, M. R. Bhatti, Z. I. Choudhary, and M. A. Cheema, "A survey of Autism knowledge and attitudes among the healthcare professionals in Lahore, Pakistan," BMC Pediatr., vol. 11, Nov. 2011.

[46] M. Banach, J. Iudice, L. Conway, and L. Couse, "Family Support and Empowerment: Post Autism Diagnosis Support Group for Parents," Soc. Work Groups, vol. 33, pp. 69–83, Jan. 2010.

[47] M. A. Al-zaalah, A. H. Al-asmari, H. H. Al-malki, N. M. Al-shehri, N. M. Almoalwi, and O. A. Mostafa, "Characteristics of Autism Spectrum Disorder among Saudi Children and its Impact on their Families," 2015.

[48] P. Sah, E. S. L. Faber, M. L. De Armentia, and J. Power, "The amygdaloid complex: Anatomy and physiology," Physiological Reviews, vol. 83, no. 3. American Physiological Society, pp. 803–834, 2003.

[49] R. Adolphs, "What does the amygdala contribute to social cognition?," Annals of the New York Academy of Sciences, vol. 1191, no. 1. Blackwell Publishing Inc., pp. 42–61, 2010.

[50] J. C. Marshall and G. M. Morriss-Kay, "Functional anatomy of the human brain," Journal of Anatomy, vol. 205, no. 6. Wiley-Blackwell, p. 415, Dec-2004.

[51] M. L. Bauman and T. L. Kemper, "Structural brain anatomy in Autism: What is the evidence?," in The Neurobiology of Autism, 2005, pp. 121–135.

[52] A. Collins and E. Koechlin, "Reasoning, Learning, and Creativity: Frontal Lobe Function and Human Decision-Making," PLoS Biol., vol. 10, no. 3, p. e1001293, Mar. 2012.

[53] T. E. Schlaepfer et al., "Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression," Neuropsychopharmacology, vol. 33, no. 2, pp. 368–377, Jan. 2008.

[54] R. A. Fuchs, J. L. Eaddy, Z. I. Su, and G. H. Bell, "Interactions of the basolateral amygdala with the dorsal hippocampus and dorsomedial prefrontal cortex regulate drug context-induced reinstatement of cocaine-seeking in rats," Eur. J. Neurosci., vol. 26, no. 2, pp. 487–498, Jul. 2007.

[55] M. X. Cohen, N. Axmacher, D. Lenartz, C. E. Elger, V. Sturm, and T. E. Schlaepfer, "Neuroelectric signatures of reward learning and decision-making in the human nucleus accumbens," Neuropsychopharmacology, vol. 34, no. 7, pp. 1649–1658, Jun. 2009.

[56] T. E. Schlaepfer et al., "Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression," Neuropsychopharmacology, vol. 33, no. 2, pp. 368–377, Jan. 2008.

[57] A. A. Scott-Van Zeeland, M. Dapretto, D. G. Ghahremani, R. A. Poldrack, and S. Y. Bookheimer, "Reward processing in autism," Autism Res., vol. 3, no. 2, pp. 53–67, Apr. 2010.

[58] D. Skuse et al., "The Developmental, Dimensional and Diagnostic Interview (3di): A Novel Computerized Assessment for Autism Spectrum Disorders," J. Am. Acad. Child Adolesc. Psychiatry, vol. 43, no. 5, pp. 548–558, 2004.

[59] D. Neal, J. L. Matson, and M. A. Hattier, "Validity of the Autism Spectrum Disorder Observation for Children (ASD-OC)," J. Ment. Health Res. Intellect. Disabil., vol. 7, no. 1, pp. 14–33, 2014.

[60] C. Lord, M. Rutter, and A. Le Couteur, "Autism Diagnostic Interview-Revised: A revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders," J. Autism Dev. Disord., vol. 24, no. 5, pp. 659–685, Oct. 1994.

[61] C. Gillberg, C. Gillberg, M. Råstam, and E. Wentz, "The Asperger Syndrome (and high-functioning autism) Diagnostic Interview (ASDI): A preliminary study of a new structured clinical interview," Autism, vol. 5, no. 1, pp. 57–66, 2001.

[62] J. Maljaars, I. Noens, E. Scholte, and I. Van Berckelaer-Onnes, "Evaluation of the criterion and convergent validity of the Diagnostic Interview for Social and Communication Disorders in young and low-functioning children," Autism, vol. 16, no. 5, pp. 487–497, 2012.

[63] S. Khadka et al., "Multivariate imaging genetics study of MRI gray matter volume and SNPs reveals biological pathways correlated with brain structural differences in attention deficit hyperactivity disorder," Front. Psychiatry, vol. 7, no. JUL, 2016.

[64] T. Frodl and N. Skokauskas, "Meta-analysis of structural MRI studies in children and adults with attention deficit hyperactivity disorder indicates treatment effects," Acta Psychiatr. Scand., vol. 125, no. 2, pp. 114–126, Feb. 2012.

[65] S. LeClerc and D. Easley, "Pharmacological therapies for autism spectrum disorder: A review," P T, vol. 40, no. 6, pp. 389–397, Jun. 2015.

[66] Y. Zhang et al., "Decreased brain levels of vitamin B12 in aging, autism and schizophrenia," PLoS One, vol. 11, no. 1, Jan. 2016.

[67] A. O. Caglayan, "Genetic causes of syndromic and non-syndromic autism," Developmental Medicine and Child Neurology, vol. 52, no. 2. pp. 130–138, Feb-2010.

[68] D. R. Morales, J. Slattery, S. Evans, and X. Kurz, "Antidepressant use during pregnancy and risk of autism spectrum disorder and attention deficit hyperactivity disorder: Systematic review of observational studies and methodological considerations," BMC Med., vol. 16, no. 1, 2018.

[69] X. Li, H. Zou, and W. T. Brown, "Genes associated with autism spectrum disorder," Brain Research Bulletin, vol. 88, no. 6. Brain Res Bull, pp. 543–552, 01-Sep-2012.

[70] M. Alarcón et al., "Linkage, Association, and Gene-Expression Analyses Identify CNTNAP2 as an Autism-Susceptibility Gene," Am. J. Hum. Genet., vol. 82, no. 1, pp. 150–159, Jan. 2008.

[71] K. Wang et al., "Common genetic variants on 5p14.1 associate with autism spectrum disorders," Nature, vol. 459, no. 7246, pp. 528–533, May 2009.

[72] J. T. Glessner et al., "Autism genome-wide copy number variation reveals ubiquitin and neuronal genes," Nature, vol. 459, no. 7246, pp. 569–572, May 2009.

[73] R. Anney et al., "A genome-wide scan for common alleles affecting risk for autism," Hum. Mol. Genet., vol. 19, no. 20, pp. 4072–4082, Jul. 2010.

[74] S. C. Cho et al., "Genome-wide association scan of Korean autism spectrum disorders with language delay: A preliminary study," Psychiatry Investig., vol. 8, no. 1, pp. 61–66, Mar. 2011.

[75] S. Gentile, "Risks of neurobehavioral teratogenicity associated with prenatal exposure to valproate monotherapy: A systematic review with regulatory repercussions," CNS Spectrums, vol. 19, no. 4. Cambridge University Press, pp. 305–315, 2014.

[76] T. Kobayashi, T. Matsuyama, M. Takeuchi, and S. Ito, "Autism spectrum disorder and prenatal exposure to selective serotonin reuptake inhibitors: A systematic review and meta-analysis," Reprod. Toxicol., vol. 65, pp. 170–178, Oct. 2016.

[77] L. E. Taylor, A. L. Swerdfeger, and G. D. Eslick, "Vaccines are not associated with autism: An evidence-based meta-analysis of case-control and cohort studies," Vaccine, vol. 32, no. 29, pp. 3623–3629, Jun. 2014.

[78] H. Wei et al., "Brain IL-6 elevation causes neuronal circuitry imbalances and mediates autism-like behaviors," Biochim. Biophys. Acta - Mol. Basis Dis., vol. 1822, no. 6, pp. 831–842, Jun. 2012.

[79] R. K. Gherardi, H. Eidi, G. Crépeaux, F. J. Authier, and J. Cadusseau, "Biopersistence and brain translocation of aluminum adjuvants of vaccines," Frontiers in Neurology, vol. 6, no. FEB. Frontiers Media S.A., 2015.

[80] G. Caspani, S. Kennedy, J. A. Foster, and J. Swann, "Gut microbial metabolites in depression: understanding the biochemical mechanisms," Microb. Cell, vol. 6, no. 10, pp. 454–481, Oct. 2019.

[81] G. N. Luheshi et al., "Vagotomy attenuates the behavioural but not the pyrogenic effects of interleukin-1 in rats," in Autonomic Neuroscience: Basic and Clinical, 2000, vol. 85, no. 1–3, pp. 127–132.

[82] T. C. Fung, C. A. Olson, and E. Y. Hsiao, "Interactions between the microbiota, immune and nervous systems in health and disease," Nature Neuroscience, vol. 20, no. 2. Nature Publishing Group, pp. 145–155, 01-Feb-2017.

[83] T. Arentsen et al., "The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior," Mol. Psychiatry, vol. 22, no. 2, pp. 257–266, Feb. 2017.

[84] G. J.-S. et al., "Dose-dependent effects of endotoxin on neurobehavioral functions in humans," PLoS One, vol. 6, no. 12, p. e28330, 2011.

[85] L. Hoyles et al., "Microbiome–host systems interactions: Protective effects of propionate upon the blood–brain barrier," Microbiome, vol. 6, no. 1, 2018.

[86] L. Galland, "The gut microbiome and the brain," Journal of Medicinal Food, vol. 17, no. 12. Mary Ann Liebert Inc., pp. 1261–1272, 01-Dec-2014.

[87] P. Forsythe, J. Bienenstock, and W. A. Kunze, "Vagal pathways for microbiome-brain-gut axis communication," Adv. Exp. Med. Biol., vol. 817, pp. 115–133, 2014.

[88] E. S. Chambers et al., "Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults," Gut, vol. 64, no. 11, pp. 1744–1754, Nov. 2015.

[89] L. Wang, C. T. Christophersen, M. J. Sorich, J. P. Gerber, M. T. Angley, and M. A. Conlon, "Increased abundance of Sutterella spp. and Ruminococcus torques in feces of children with autism spectrum disorder," Mol. Autism, vol. 4, no. 1, 2013.

[90] K. P. Ossenkopp et al., "Systemic treatment with the enteric bacterial fermentation product, propionic acid, produces both conditioned taste avoidance and conditioned place avoidance in rats," Behav. Brain Res., vol. 227, no. 1, pp. 134–141, Feb. 2012.

[91] R. Mitsui, S. Ono, S. Karaki, and A. Kuwahara, "Neural and non-neural mediation of propionate-induced contractile responses in the rat distal colon," Neurogastroenterol. Motil., vol. 17, no. 4, pp. 585–594, Aug. 2005.

[92] M. DeCastro et al., "Short chain fatty acids regulate tyrosine hydroxylase gene expression through a cAMP-dependent signaling pathway," Mol. Brain Res., vol. 142, no. 1, pp. 28–38, Dec. 2005.

[93] M. C. P. Cleophas et al., "Suppression of monosodium urate crystal-induced cytokine production by butyrate is mediated by the inhibition of class I histone deacetylases," Ann. Rheum. Dis., vol. 75, no. 3, pp. 593–600, Mar. 2016.

[94] T. E. Davis et al., "Anxiety symptoms across the lifespan in people diagnosed with Autistic Disorder," Res. Autism Spectr. Disord., vol. 5, no. 1, pp. 112–118, Jan. 2011.

[95] Y. Leitner, "The co-occurrence of autism and attention deficit hyperactivity disorder in children - What do we know?," Front. Hum. Neurosci., vol. 8, no. 1 APR, Apr. 2014.

[96] P. A. Rao and R. J. Landa, "Association between severity of behavioral phenotype and comorbid attention deficit hyperactivity disorder symptoms in children with autism spectrum disorders," Autism, vol. 18, no. 3, pp. 272–280, 2014.

[97] T. Chandrasekhar and L. Sikich, "Challenges in the diagnosis and treatment of depression in autism spectrum disorders across the lifespan," Dialogues Clin. Neurosci., vol. 17, no. 2, pp. 219–227, 2015.

[98] E. Simonoff, A. Pickles, T. Charman, S. Chandler, T. Loucas, and G. Baird, "Psychiatric disorders in children with autism spectrum disorders: Prevalence, comorbidity, and associated factors in a population-derived sample," J. Am. Acad. Child Adolesc. Psychiatry, vol. 47, no. 8, pp. 921–929, 2008.

[99] S. D. Mayes, S. L. Calhoun, M. J. Murray, M. Ahuja, and L. A. Smith, "Anxiety, depression, and irritability in children with autism relative to other neuropsychiatric disorders and typical development," Res. Autism Spectr. Disord., vol. 5, no. 1, pp. 474–485, Jan. 2011.

[100] T. Lugnegård, M. U. Hallerbäck, and C. Gillberg, "Psychiatric comorbidity in young adults with a clinical diagnosis of Asperger syndrome," Res. Dev. Disabil., vol. 32, no. 5, pp. 1910–1917, Sep. 2011.

[101] O. T. Leyfer et al., "Comorbid psychiatric disorders in children with autism: Interview development and rates of disorders," J. Autism Dev. Disord., vol. 36, no. 7, pp. 849–861, Oct. 2006.

[102] A. J. Russell, D. Mataix-Cols, M. Anson, and D. G. M. Murphy, "Obsessions and compulsions in Asperger syndrome and high-functioning autism," Br. J. Psychiatry, vol. 186, no. JUNE, pp. 525–528, Jun. 2005.

[103] G. Joshi, J. Biederman, C. Petty, R. L. Goldin, S. L. Furtak, and J. Wozniak, "Examining the comorbidity of bipolar disorder and autism spectrum disorders: A large controlled analysis of phenotypic and familial correlates in a referred population of youth with bipolar i disorder with and without autism spectrum disorders," J. Clin. Psychiatry, vol. 74, no. 6, pp. 578–586, 2013.

[104] T. Munesue, Y. Ono, K. Mutoh, K. Shimoda, H. Nakatani, and M. Kikuchi, "High prevalence of bipolar disorder comorbidity in adolescents and young adults with high-functioning autism spectrum disorder: A preliminary study

of 44 outpatients," J. Affect. Disord., vol. 111, no. 2–3, pp. 170–175, Dec. 2008.

[105] L. Burd, Q. Li, J. Kerbeshian, M. G. Klug, and R. D. Freeman, "Tourette Syndrome and Comorbid Pervasive Developmental Disorders," J. Child Neurol., vol. 24, no. 2, pp. 170–175, Feb. 2009.

[106] D. I. Zafeiriou, A. Ververi, and E. Vargiami, "Childhood autism and associated comorbidities," Brain and Development, vol. 29, no. 5. pp. 257–272, Jun-2007.

[107] Q. Wang and R. Xu, "Data-driven multiple-level analysis of gut-microbiome-immune-joint interactions in rheumatoid arthritis," BMC Genomics, vol. 20, no. 1, p. 124, Dec. 2019.

[108] M. Kuhn, C. von Mering, M. Campillos, L. J. Jensen, and P. Bork, "STITCH: Interaction networks of chemicals and proteins," Nucleic Acids Res., vol. 36, no. SUPPL. 1, Jan. 2008.

[109] C. von Mering et al., "STRING: Known and predicted protein-protein associations, integrated and transferred across organisms," Nucleic Acids Res., vol. 33, no. DATABASE ISS., Jan. 2005.

[110] A. P. Davis et al., "The Comparative Toxicogenomics Database: Update 2019," Nucleic Acids Res., vol. 47, no. D1, pp. D948–D954, Jan. 2019.

[111] Y. C. Lin, C. C. Wang, and C. W. Tung, "An in silico toxicogenomics approach for inferring potential diseases associated with maleic acid," Chem. Biol. Interact., vol. 223, pp. 38–44, Nov. 2014.

[112] T. S. Carpenter, D. A. Kirshner, E. Y. Lau, S. E. Wong, J. P. Nilmeier, and F. C. Lightstone, "A Method to Predict Blood-Brain Barrier Permeability of Drug-Like Compounds Using Molecular Dynamics Simulations," Biophys. J., vol. 107, no. 3, pp. 630–641, Aug. 2014.

[113] H. Pajouhesh and G. R. Lenz, "Medicinal chemical properties of successful central nervous system drugs," NeuroRx, vol. 2, no. 4, pp. 541–553, 2005.

[114] H. Yang et al., "AdmetSAR 2.0: Web-service for prediction and optimization of chemical ADMET properties," Bioinformatics, vol. 35, no. 6, pp. 1067–1069, Mar. 2019.

[115] M. Chagoyen, J. López-Ibáñez, and F. Pazos, "Functional analysis of metabolomics data," in Methods in Molecular Biology, vol. 1415, Humana Press Inc., 2016, pp. 399–406.

[116] M. Kanehisa, M. Furumichi, M. Tanabe, Y. Sato, and K. Morishima, "KEGG: New perspectives on genomes, pathways, diseases and drugs," Nucleic Acids Res., vol. 45, no. D1, pp. D353–D361, Jan. 2017.

[117] A. Fabregat et al., "The Reactome Pathway Knowledgebase," Nucleic Acids Res., vol. 46, no. D1, pp. D649–D655, Jan. 2018.

[118] D. H. Ebert and M. E. Greenberg, "Activity-dependent neuronal signalling and autism spectrum disorder," Nature, vol. 493, no. 7432. Nature, pp. 327–337, 17-Jan-2013.

[119] S. Guang et al., "Synaptopathology involved in autism spectrum disorder," Frontiers in Cellular Neuroscience, vol. 12. Frontiers Media S.A., 21-Dec-2018.

[120] V. Verma, A. Paul, A. A. Vishwanath, B. Vaidya, and J. P. Clement, "Understanding intellectual disability and utism spectrum disorders from common mouse models: Synapses to behaviour," Open Biol., vol. 9, no. 6, Jun. 2019.

[121] C. L. Muller, A. M. J. Anacker, and J. Veenstra-VanderWeele, "The serotonin system in autism spectrum disorder: From biomarker to animal models," Neuroscience, vol. 321. Elsevier Ltd, pp. 24–41, 2016.

[122] M. Nguyen et al., "Decoding the contribution of dopaminergic genes and pathways to autism spectrum disorder (ASD)," Neurochemistry International, vol. 66, no. 1. pp. 15–26, Jan-2014.

[123] D. Pavăl, "A Dopamine Hypothesis of Autism Spectrum Disorder," Dev. Neurosci., vol. 39, no. 5, pp. 355–360, Sep. 2017.

[124] K. Tabata, T. Yoshida, and J. Naoe, "P-4 * THREE CASES OF ALCOHOLISM WITH AUTISM SPECTRUM DISORDER," Alcohol Alcohol., vol. 49, no. suppl 1, pp. i54–i54, Sep. 2014.

[125] A. Meltzer and J. Van De Water, "The Role of the Immune System in Autism Spectrum Disorder," Neuropsychopharmacology, vol. 42, no. 1. Nature Publishing Group, pp. 284–298, 01-Jan-2017.

[126] A. P. Gabrielli, A. M. Manzardo, and M. G. Butler, "GeneAnalytics pathways and profiling of shared autism and Cancer Genes," Int. J. Mol. Sci., vol. 20, no. 5, Mar. 2019.

[127] H. Wang et al., "Potential serum biomarkers from a metabolomics study of autism," J. Psychiatry Neurosci., vol. 41, no. 1, pp. 27–37, Jan. 2016.

[128] K. Ohja et al., "Neuroimmunologic and Neurotrophic Interactions in Autism Spectrum Disorders: Relationship to Neuroinflammation," NeuroMolecular Medicine, vol. 20, no. 2. Humana Press Inc., pp. 161–173, 01-Jun-2018.

[129] H. Wei, I. Alberts, and X. Li, "The apoptotic perspective of autism," Int. J. Dev. Neurosci., vol. 36, pp. 13–18, 2014.

[130] H. Yamasue and G. Domes, "Oxytocin and autism spectrum disorders," in Current Topics in Behavioral Neurosciences, vol. 35, Springer Verlag, 2018, pp. 449–465.

[131] J. Vithayathil, J. Pucilowska, and G. E. Landreth, "ERK/MAPK signaling and autism spectrum disorders," in Progress in Brain Research, vol. 241, Elsevier B.V., 2018, pp. 63–112.

[132] A. Nadeem et al., "Toll-like receptor 4 signaling is associated with upregulated NADPH oxidase expression in peripheral T cells of children with autism," Brain. Behav. Immun., vol. 61, pp. 146–154, Mar. 2017.

[133] Y. Wen, M. J. Alshikho, and M. R. Herbert, "Pathway network analyses for autism reveal multisystem involvement, major overlaps with other diseases

and convergence upon MAPK and calcium signaling," PLoS One, vol. 11, no. 4, Apr. 2016.

[134] Y. Huang, Z. Chang, X. Li, S. Liang, Y. Yi, and L. Wu, "Integrated multi-factor analysis explores core dysfunctional modules in autism spectrum disorder," Int. J. Biol. Sci., vol. 14, no. 8, pp. 811–818, May 2018.

[135] P. Srikantha and M. Hasan Mohajeri, "The possible role of the microbiota-gut-brain-axis in autism spectrum disorder," Int. J. Mol. Sci., vol. 20, no. 9, May 2019.

[136] K. E. Glinton and S. H. Elsea, "Untargeted Metabolomics for Autism Spectrum Disorders: Current Status and Future Directions," Frontiers in Psychiatry, vol. 10. Frontiers Media S.A., 10-Sep-2019.

[137] I. K. S. Yap, M. Angley, K. A. Veselkov, E. Holmes, J. C. Lindon, and J. K. Nicholson, "Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls," J. Proteome Res., vol. 9, no. 6, pp. 2996–3004, Jun. 2010.

[138] F. Gevi, L. Zolla, S. Gabriele, and A. M. Persico, "Urinary metabolomics of young Italian autistic children supports abnormal tryptophan and purine metabolism," Mol. Autism, vol. 7, no. 1, p. 47, Dec. 2016.

[139] D. Lonsdale, R. J. Shamberger, and M. E. Obrenovich, "Dysautonomia in Autism Spectrum Disorder: Case Reports of a Family with Review of the Literature," Autism Res. Treat., vol. 2011, pp. 1–7, 2011.

[140] R. Xu and Q. Q. Wang, "Towards understanding brain-gut-microbiome connections in Alzheimer's disease," BMC Syst. Biol., vol. 10, no. 3, p. 63, Aug. 2016.

[141] S. V Gondalia, P. J. Mahon, E. A. Palombo, S. R. Knowles, and D. W. Austin, "Evaluation of Biogenic Amines in the Faeces of Children with and without Autism by LC-MS/MS," International Journal of Biotechnology and Biochemistry, vol. 9, no. 2, pp. 245-255, 2013.

[142] B. L. Williams et al., "Impaired Carbohydrate Digestion and Transport and Mucosal Dysbiosis in the Intestines of Children with Autism and Gastrointestinal Disturbances," PLoS One, vol. 6, no. 9, p. e24585, Sep. 2011.

[143] A. Kusaga, "[Decreased beta-phenylethylamine in urine of children with attention deficit hyperactivity disorder and autistic disorder].," No to hattatsu = Brain Dev., vol. 34, no. 3, pp. 243–8, May 2002.

[144] S. Liu et al., "Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder," Sci. Rep., vol. 9, no. 1, pp. 1–9, Dec. 2019.

[145] A. M. Persico and V. Napolioni, "Urinary p-cresol in autism spectrum disorder," Neurotoxicol. Teratol., vol. 36, pp. 82–90, Mar. 2013.

[146] S. Gabriele et al., "Urinary p-cresol is elevated in young French children with autism spectrum disorder: A replication study," Biomarkers, vol. 19, no. 6, pp. 463–470, 201