CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Elucidation of Pathway Crosstalks and Prospective Therapeutic Targets in Autism Spectrum Disorder

by

Umm-e-Farwa

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

2021

I dedicate this thesis to my parents and my teachers.

CERTIFICATE OF APPROVAL

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Acknowledgement

In the name of Allah, the Most Gracious and the Most Merciful.

Alhamdulillah, there are no words for expressing gratitude to ALMIGHTY ALLAH who has bestowed me with more than I deserve. All praises to ALLAH for giving me strength and ability to complete this work. All respect to the holy Prophet (Peace Be Upon Him) and his family who enabled us to recognize our creator and make us able to complete this work.

I am unable to find words for expressing my feelings towards my supervisor **Dr. Syeda Marriam Bakhtiar**, Assistant professor, Faculty of Health and Life Sciences, Department of Bioinformatics and Biosciences, Capital University of Science and Technology, Islamabad for her sincere guidance, suggestions and trust in me throughout my thesis research. Her comments makes me able to organize my work, and establish overall direction of research. I cannot thank her enough for her unconditional support and guidance throughout the research.

I would like to express my sincere gratitude to Capital University of Science and Technology (CUST) Islamabad for providing me an opportunity to do MS Biosciences and achieving my goal to pursue higher studies. I owe a great deal of appreciation and gratitude to my Head Of Department Dr. Sahar Fazal for providing us the necessary facilities required for research activities. I want to pay my gratitude to other faculty members Dr. Shaukat Iqbal, Dr. Erum Dilshad and Dr. Arshia Amin for their enlightenment and guidance throughout the degree.

In the end, I gratefully acknowledge and special thank my family for their contribution, love, care, support, prayers and encouragement. I would like to thanks and pay my gratitude to my friends and colleagues specially Muhammad Maaz

& Ayesha Safdar for supporting and guiding me throughout this time. Finally, I express my gratitude to my parents and siblings who have always enlightened my way throughout my life. May Allah bless them all.

(Umm-e-Farwa)

Abstract

Autism spectrum disorder is a group of heterogeneous neurodevelopmental disorders, which lies under the category of pervasive developmental disorder as an autistic disorder. It also includes some other diseases like Asperger's disorder, Rett's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS). DSM-5 changes the criteria for diagnostic purposes by terminating the above diseases. ASD core symptoms are impairments of social interaction, restricted and stereotyped behavior, and delay in language. Limited treatment options are available to cure the core symptoms of ASD. In this study in-silico approaches were used to find out the cross talks among major pathways involved in autism including developmental, pro inflammatory and metabolic pathways. For this purpose key pathways were identified from literature. Secondly, microarray data of autistic patients have been used resulting in differentially expressed genes. Identification of gene ontology and pathway enrichment of these DEGs had been done to know the enriched genes involved in ASD. Further, protein protein interactions have been identified and validated through the KEGG database. Finally, cross talks of pathways were generated identifying 10 pathways crosstalks in ASD, among which few were reported in literature and other were identified as novel pathways. Their cross-talks are important for finding and predicting actual potential targets or biomarkers which by further investigations could be the novel approach of diagnosis or treatment. Keywords: Autism spectrum disorder, neurodevelopmental disease, DEGs, pathway cross talks.

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Contents

List of Figures

List of Tables

Abbreviations

ASD	Autism spectrum disorder
APA	American Psychiatric Association
ADHD	Attention Deficit hyperactivity disorder
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
DEG	Differentially Expressed Genes
FDA	Food and Drug Administration
FQS	Friendship Qualities Scales
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
NCI	National Cancer Institute
PEERS	Program for Education and Enrichment of Relational Scales
PDD	Pervasive developmental disorders
PDD-NOS	pervasive developmental disorder not otherwise specified
QSQ	Quality of Socialization Questionnaire
SSRS	Social Skills Rating System

Chapter 1

Introduction

1.1 Background

Autism Spectrum disorder (ASD) is neurodevelopmental, heterogeneous disorder lies under the category of pervasive developmental disorder characterized by impaired communications and interactions as well as repetitive behaviors [1]. Pervasive developmental disorders (PDD) are a group of neurodevelopmental heterogeneous disorders. It is characterized by poor communication interactions, a wide range of limitations and interests, social and communication difficulties and stereotyped or repetitive behavior [2].

According to the Diagnostic and Statistical Manual of Mental Disorders, system 4 (DSM-IV) PDD includes autistic disorder (autism), Asperger's disorder, pervasive developmental disorder not otherwise specified (PDD-NOS), and Rett's disorder. DSM fifth edition (DSM-V) introduced notable changes, eradicating the diagnostic subtypes of PDD [3,4].

These disorders have common features but are altered at specific symptoms. They are considered to be lying on the spectrum with autism and given the name autism spectrum disorder [5]. ASD is one of the groups of PDD comprising the same characteristics and features such as lack of social interactions, language delay, reciprocal behavior [6]. ASD manifests within the first three years of life [7].

Research has highlighted the social and cultural variables for diagnostic criteria, typical symptoms are in US developmental delyas and delay in language skills. Language is not taken into account in diagnostic criteria in India, as boys learn language skills later than girls.

In Asian cultures, direct eye contact with elders is considered a sign of disrespect, so decreased "eye contact" may be less observed in these cultures as a diagnostic feature. Researchers must therefore have a diagnostic platform that discusses cross-cultural factors for the diagnosis of ASD [8]. This term ASD has been used to explain its variable presentation [2].

The main causes of ASD are yet unknown, some pieces of evidence manifest that the etiology of ASD varies from genetic to environmental factors [9]. In many brain regions, including the frontal cortex, hippocampus, cerebellum and amygdaloid nucleus and cerebellothalamo-cortical pathways, post-mortem and neuroimaging studies with ASD animals have shown abnormalities. In ASD, neuronal migration in the brain limits certain focal points of thin cortical areas, particularly in the frontal lobe. In ASDD, the average brain size is similarly increased [10].

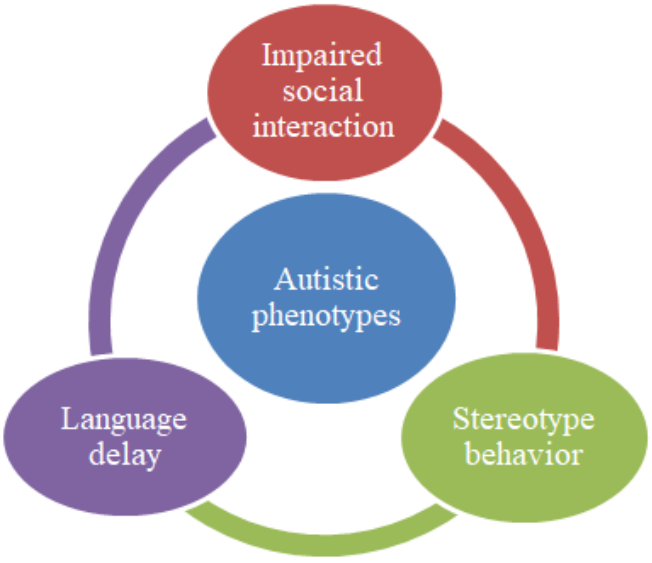

FIGURE 1.1: DSM-5 criteria to recognize the core symptoms of ASD for diagnosis purpose [8].

The prevalence of ASD is difficult, because of multiple changes in criteria for diagnosis all over the world, heterogeneity of the disease, and the absence of data

collection strategies [11]. In Asia, the area of South Asia makes up about 20 percent of the world's population, but the prevalence of ASD is still not understood. Eight countries are located in these areas, including Bangladesh, India, Pakistan, Nepal, Sri Lanka, Bhutan, the Maldives and Afghanistan. The prevalence of ASD in Bangladesh and India was stated in several studies to be 0.84 percent and 0.23 percent, respectively [12]. In Pakistan, no specific and reliable data is so far generated [13,14,9]. The overall estimated prevalence of ASD in south Asia is 1.4 % [15]. The worldwide rate of ASD is higher in males than females that are 4/1 ratio [10]. 1% to 2% of children are affected by ASD in the US [1]. The diagnosis of ASD depends on how the children show their atypical behavior, lack of communication, and stereotyped behavior [8]. Defective linguistic skills and delayed development are considered as common symptoms for early diagnosis. Hence, for measurements of impaired communication and atypical behavior some measuring scales has been used such as the Program for Education and Enrichment of Relational Scales (PEERS), which includes Friendship Qualities Scales (FQS), Social Skills Rating System (SSRS), Quality of Socialization Questionnaire (QSQ) [16].

The criteria for efficient diagnosis of ASD are included in the DSM that the American Psychiatric Association publishes (APA). For diagnosis of autistic disorder, it is essential to have at least six symptoms out of 12 from three domains, these domains include two social, communicational, and at least one behavioral. But according to DSM-V individual with ASD must specify all three social criteria, (that is the lack in social-emotional interaction, lack in non-verbal communicative behavior, and absence of developing, understanding, and maintaining relationships) and two of four behavior criteria (that is repetitive speech, demanding behavior, lack of interest, and abnormal response to sensory input) [17].

Early and efficient diagnosis helps the family as well as professionals to know about the child's developmental abnormalities and finding suitable services for ASD patients [18]. Unfortunately, ASD patients are not diagnosed until their school age. Whereas patients with less severity are even diagnosed later [1]. Diagnosis of ASD is possible at 14 months but most children are diagnosed after two years of age [19]. For the treatment of ASD, no evidence-based treatment is present yet. There are

limited FDA (Food and Drug Administration) approved medicines, which targets the core symptoms [20]. Hence, various challenges are faced by the researchers for finding effective and efficient treatment. Two FDA-approved drugs namely risperidone and aripiprazole are used for ASD treatment which blocks the brain receptors for dopamine and serotonin improving the symptoms of ASD [8,21]. Many brain neurotransmitters play a role in ASD initiation and development [21].

Divalproex is also an FDA approved drug for epilepsy, migraine, and bipolar disorders which help in improving repetitive behavior, use of this drug would help in improvement in the behavioral pattern by blocking sodium ions channels, inhibiting glutamine, and acting on serotonin [22]. There is no specific cure for ASD so far, and patients need further treatment and care. Future research is critically demanding for better treatment of ASD patients [23].

Biological pathways or wiring diagrams express some biomolecules and gene interactions together with their relationships corresponding to some biological pathways. Pathways Analysis (PA) algorithms are a set of widely used tools, which are interlinked with large molecular databases for processing of high-throughput genomics or proteomics data [24,25].

PA manifests crucial importance, because of its importance more than 70 methods of pathway analysis have already been proposed; they are primarily divided into two major groups. The first group comprises a group that includes "non-topology-based method" also called "gene set analysis method", it contains two generations analysis tools, firstly, over-representation analysis (ORA) having affinity to identify those pathways in which genes are up or down-regulated, secondly functional class scoring method (FCS) it abolishes the dependencies on gene assortment criteria.

The second group contains the "topology-based method" which includes all the dependencies and interactions among genes [26]. The current challenges in PA are annotation and methodological challenges [27]. In PA the P-value shows its significance in calculating the PA phenotypes. Moreover, pathways can affect each other's P-value which is termed as cross-talks. These cross talks occur due to interactions of biomolecules, or gene overlapping among the pathways [28]. Multiple

online databases are present to form and analysis of pathways, including the Kyoto Encyclopaedia of Genes and Genome (KEGG), Reactome, GenMAPP, and BioCarta [24]. Most PA tools rely on KEGG due to large-scale literature analysis and consistent updating. Biological pathways involve more than one pathway forming a network, such networks cannot be obtained from literature easily because the network structure is dynamics and alter with the environment [29].

The development of gene-disease associations will enhance the production of new strategies for disease diagnosis, prevention and treatment. It takes more time and effort to distinguish disease genes from hundreds of candidate genes through experiments. In this regard, the approach of network analysis using bioinformatics techniques is used to extract identified disease genes and to predict unknown genes that may be good candidates for experimental purposes [30].

The foremost issue in biology is to identify disease genes and pathways. Gene's interaction network makes it easy to describe which genes are interconnected within the given pathway [31].

In networks, the genes representing the nodes, while the edges represent functional relationships among genes. Hub protein/genes are considered to be master genes, hence we choose that specific gene and network for highlighting the most relevant network, by selecting highly connected nodes with nearest edges [32]. Bioinformatics study of these high-performance data highlighted complex biological networks for therapeutic target recognition [33].

Network analysis is used to identify disease-related genes. For network analysis many tools would be used, Cytoscape network analyzer is one of the tools used for network analysis. It is used for the construction of subnetworks which are disease-related networks [34]. Pathway cross talks may manifest better ways for effective drug treatment of autism to improve the disease condition. Consequently, identifying molecular targets is vital for elucidation of pathway crosstalks and prospective and better therapeutic interventions [35]. The cell responds to its environment through signaling pathways, it consists of many regulatory proteins as well as interactions among cells receptors, intermediate protein complex,

transcription factors and target genes. When the external stimulus initiates the receptor some conformational changes occur to intermediate protein which activates the target genes by regulation of transcriptional factors. This stimulation of one pathway may affect the target gene of other pathway, this interlinked connections of pathways is given the name "pathway cross talks". It imparts great effect it developmental processes. Atypical regulation of pathway crosstalk has been associated to neurodegeneration [36]. Pathway cross talk describes communication between the pathways because biomolecules involved perform more than one part in particular pathway so it can engage in more than one biological role [37]. Major pathway crosstalks in ASD are divided to developmental, metabolic and pro inflammatory and cytokine pathways.

1.2 Aims and Objectives

There is no proper literature on pathway crosstalk involved in autism, the pathways reported in the literature are diverse, and there is no interlinking among these pathways. Therefore, no particular treatment strategies for autism are available. Identifications of interactions of pathways for a better understanding of autism involved genes; it may contribute to better prevention and treatment. Generally, previous studies mainly focused on disrupted pathways between normal and disease, or their common genes between two pathways.

Pathway cross talks enable one to explore better prevention and therapeutic ways by inhibiting certain interactions among pathways using bioinformatics tools. Computational techniques or bioinformatics decrease men's effort, time, and cost of the experiment. In this study by using bioinformatics tools network interaction can be measured with the analysis and crosstalk of those pathways for autism spectrum disorder.

It will lead towards identifying those genes which are involved in cross-talks and by targeting those genes treatment strategies could be developed in better ways. Autism spectrum disorder is a multifactorial disease which would be an outcome

of variation or disorders in multiple pathways. This study is design with an aim to analyse these pathways in order to establish a cross talk among major pathways. In order to achieve the aim, study is divided in following objectives.

1. Prioritising of key pathways reported in literature to be involved in autism spectrum disorder based on pathway crosstalks .
2. Identification of key genes involved in autism using pathway cross talks.
3. Elucidation of therapeutic targets based pathway cross talks.

Chapter 2

Literature Review

2.1 Autism Spectrum Disorder

Autism spectrum disorder is a group of major neurodevelopmental disorders, which lies under the category of pervasive developmental disorder as an autistic disorder. It also includes some other diseases like Asperger's disorder, Rett's disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) [2].

PDD are characterized by poor communication skills, impaired social interactions, lack of interest and repetitive behaviour.

The term spectrum has been used for explaining variability characters among disorders and are considered to be lying on spectrum and hence given the name Autism spectrum disorder.

DSM-5 changes the criteria for diagnostic purposes by terminating the above diseases. ASD core symptoms are social contact impairments, restricted or stereotyped behavior, and delay in language [10].

Normally, it manifests at an early age, first three years of life but the DSM-5 diagnostic criteria have not noted the age of onset of ASD [7]. Heterogeneity in ASD contributes to genetic variability, comorbidity, and gender. Some study illustrates ASD's heterogeneous actions by specifying ASD-related medical and

pathological comorbidities such as mitochondrial dysfunction, sleep disturbances, epilepsy, depression, and anxiety [8].

2.2 Classification of ASD

2.2.1 Classification Based on Diagnosis

According to the Diagnostic and Statistical Manual of Mental Disorders Program 4 (DSM-IV) widespread dementia includes autism spectrum disorder (autism), Asperger's syndrome, pervasive developmental disorder not otherwise specified (PDD-NOS) and Rett's disorder. DSM fifth edition (DSM-V) have notable changes, eradicating the diagnostic subtypes of PDD [3,4].

According to DSM-V, diagnosis of ASD is different with another disorder characterized impaired brain development [11]. They are considered to be lying on the spectrum with autism and given the name autism spectrum disorder [5].

Due to wide variations of symptoms within and across the groups, DSM-V shifted these groupings under the umbrella of PDD as members of broader category of ASD.

However, researchers compared the specificity rates of DSM-IV and DSM-V in ASD diagnosis, reporting that 91 percent of patients using DSM-IV reported as ASD retained diagnosis when using DSM-V [38].

2.2.2 Classification Based on Pathogenicity

ASD can be classified into (a) syndromic (b) non-syndromic or idiopathic. Syndromic ASD refers typically, syndromic ASD refers to a condition with additional phenotypes or dysmorphic symptoms associated with chromosome defects, genetic aberration or single mutation in genes, for instance 'rett syndrome and fragile X syndrome (FXS)'. These monogenic conditions are uncommon, and more than 1-2

percent of ASD cases can be explained by none of these genes, but together they account for approximately less than 5% of ASD cases [39]. 90 percent of FXS male patients have certain ASD characteristics, while up to 60 percent meet the ASD diagnostic criteria [11]. It takes place in the 5 'untranslated region of the fragile X mental retardation gene (FMR1) located at Xq27.33 due to the expansion of trinucleotide CGG. In FXS patients, about 90% of male children have more than one symptoms of autism (e.g. atypical social interaction, lack of eye contact, social anxiety and avoidance, persevering speech, stereotypic actions [hand flapping], sensory stimuli hypersensitivity, impulsive aggression, or hand biting self-injurious).

Rett syndrome is an X-linked dominantly inherited neurodevelopmental disease, and is the second most common cause of cognitive impairment in women following down syndrome. It is activated by gene mutations encoding "methyl-CpG-binding protein 2 (MeCP2)"[40]. ASD is classified as non-syndromic or idiopathic, with 80 percent of cases where there is no prior correlation with other disorders being responsible for idiopathic ASD. For all the variations, they both are marked by impaired brain synaptic integration [11].

For idiopathic ASD cases, the ethiology is unknown. In the early 2000s, comparative genomic hybridization technology allowed the enrichment of a number of copies, such as 15q11-13, 16p11.2, 22q11.2, and 7q11.23, to be identified in both syndromic and non-syndromic ASD patients [39]. A de-novo balanced translocation TRPC-6 disorder is seen in a non-syndrome autistic person using multiple models, for example dental pulp cells, using neuronal cells derived from stimulated pluripotent stem cells, hence mutation reduction in TRPC6 leads to alter neuronal development, morphology and fucntions [41].

2.3 Etiology of ASD

Differentially expressed genes can cause ASD, a common example includes when the Fragile X mental retardation gene 1 (FMR1) is silenced, it leads to Fragile X

syndrome, which is usually autism [42].

26 Animal ASD models displayed dysfunctional brain's region, with the addition of cerebral cortex, temporal lobe, frontal lobe, hypothalamus, & striatum. As a pathological region in ASD,the frontal lobes, amygdala and cerebellum were highlighted by post-mortem and MIR. Seven histone genes including SNORA74A, SNORA53, SNORD17, SNORA54, SNORA74B, SNORD114-23, and RP6-206I17.3 have shown altered expressions in autistic children, indicating that histaminergic systems also change in children with autism spectrum disorder [43].

2.3.1 Genetic Factors

Investigations showed links in between ASD and genetic variation. Twin studies found the high inheritance of ASD. A polygenic basis for autistic disorder has been confirmed by linkage studies. There were few possible loci correlated with ASD in genome-wide association studies (GWAS) for ASD. In contrast, studies of copy-number variation (CNV) have proved to be more successful in distinguishing genomic regions linked to autism as well as other neurodevelopmental conditions, such as schizophrenia and epilepsy, with multiple genomic regions overlapping [44]. Since the earliest twin research, the heritability of ASD has been recognised, but 'idiopathic autism' has the potential to contribute to technological developments in genetics. This is because new mutations have been found in more than 20% of people with ASD and some severe mutations are estimated.

2.3.1.1 Copy Number Variants

Any structural chromosomal variation that causes changes to the number of the control copy, by duplications or deletions greater than 1 kb, is considered a variant of copy number (CNV)[45]. To search for deletions and duplications, whole genome scans for CNVs use methods based on genome-wide arrays. In the past year, several studies have been conducted on idiopathic ASDs. There have been many findings identified. Firstly, there were individuals (parents or siblings) with CNV

among inherited CNVs without an apparent diagnosis, consistent with the variable expressiveness of many recognized genetic disorders. Secondly, Increased de novo CNV concentrations were observed in ASDs. In one child, but not in another, some families find causal CNV, suggesting independent etiologies. At last, various recurring CNVs were present, but some CNVs seem to be etiologically exceptional, but they have only been described once [46].

2.3.1.2 Cytogenetics Studies

In around 7-8 percent of patients with ASD, cytogenetically visible chromosomal defects are found. Numeric or structural aberrations mostly affect each chromosome, but the most consistent results are fragile X and maternal 15q11-13 chromosome duplication [47]. CNVs may be deletions, duplications, inversions, or translocations. Although the position of CNVs can vary for different ASD patients, similar clinical features and outcomes may still result from these CNVs [44].

2.3.1.3 Epigenetic Dysregulation in Autism

The epigenetic defining state of chromatin controls the expression of several genes without altering sequence of primary DNA. This can involve methylation of DNA and methylation of histones and acetylation, and they may change in reaction to both genetic mutations and environmental exposure. The presence of epigenetic dysregulation in autism is suggested by many components. Mutations in genes involved in epigenetic control are caused by ASD-associated syndromes. For example, methyl-CpGbinding protein 2 (MeCP2) mutations are caused by transcriptional control abnormalities in Rett syndrome. In fact, MeCP2 binds to methylated DNA by suppressing replication of target genes. In addition, autism was associated with multiple chromosomal regions subject to parental imprinting (transcriptional management of either the maternal gene or the monoallelic expression influencing the paternal allele). In patients with autism, micro duplications

or micro deletions of region 15qllql3 associated with parental imprinting have been repeatedly reported. Similarly, autistic characteristics have been seen in women with X chromosome monosomy (X0) in Turner's syndrome, it was correlated with the X chromosome they obtained from paternal origin. Researchers have identified an interconnection of autism with single-nucleotide polymorphisms (SNP) in those genes which are specifically engaged in methylation with respect to common variants.

Recently, some direct changes in DNA methylation have been identified in lymphoblastoid cells of autistic patients, indicating decreased retinoic acid-related orphan receptor alpha gene (RORA) and B-cell lymphoma 2 gene expression (BCL-2) [48].

2.3.2 Environmental Factors

Parental age, fetal environment (e.g., sex steroids, maternal infections/immune activation, obesity, diabetes, hypertension or ultrasound tests), medications, consumption of alcohol, smoking and diet are biological environmental risk factors which are investigated for the patients of autsim.

2.3.2.1 Parental Age

Advanced paternal age is a risk factor for chromosomal abnormalities including advanced maternal age in Down's syndrome. Research reveals that in the etiology of psychological and neurodevelopmental problems, like bipolar disorder, schizophrenia, drug use disorders, ADHD, and ASD, older parental age has a significant impact. The possibility of mutations during the life cycle of spermatogenesis has been established. De novo ASD mutations, however, are more common in fathers than mothers, with several associated risk of autism in the children of aged fathers, in which DNA methylation occurs due to age factors altering their sperms. Neurobiological evidence indicates that decreased cortical thickness of the right ventral posterior cingulate cortex has been correlated with increased parental age

[48]. Likewise, at the first birth the impact of advanced age of mother or father on ASD is intensified. Recognition is important for these results because the risk of ASD may also be affected by potential causes of delayed reproductive age at the time the first child is born. First-parent age is also the biological event because problems with gamete dysfunction and fertility can delay reproduction [49].

2.3.2.2 Fetal Environment

The variables of ASD etiology have been considered for different environmental prenatal exposures present in the developing fetus. These involve changes in sex hormones, maternal obesity, diabetes, hypertension, infections and immune response, and sensitivity to ultrasound. The high-fat diet and maternal obesity effect on fetal neurodevelopment [50]. Similarly, The maternal immune system during pregnancy is triggered by infection activates cytokine signalling that passes through the placenta, which can cause various adverse neural effects in the developing fetal brain. Animal studies have associated utero ultrasound exposure with changes in neuroanatomy and function, such as in the hippocampus [48].

2.4 Impact of ASD on Public Health

The Department of Health and Human Services has been sharpening its emphasis on ASDs since 1995. Study support for autism rose fivefold from $11 to $56 million between 1995 and 2001. At the same time, new programs have been introduced by both the CDC and the National Institutes of Health (NIH), creating a federal basis for a public health response to autism. Catalyzed by the 2000 Children's Health Act, 71, the CDC encourages the implementation of population-based ASD monitoring programs in eight states and has supported five autism and intellectual disorder epidemiology centers of excellence to perform surveillance and to launch a broad, multi-centered autism case control study. In view of the differences in information about the stress, triggers, and most productive management methods for ASDs, this seems perfectly necessary. We hope this will contribute to the evidence

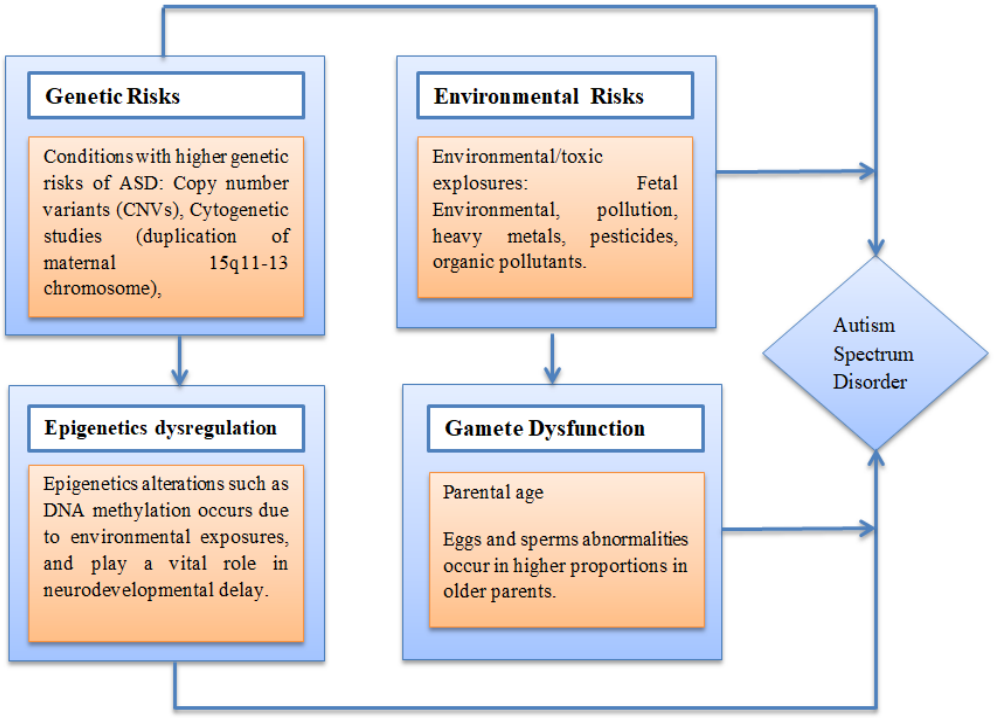

FIGURE 2.1: The link between genetic and environmental factors, In the ethiology of ASD [49].

base required to help the future of a more knowledgeable and systematic approach to autism in public health [51]. While various economically established countries, such as those in Western Europe and North America,are currently benefiting from increased understanding, detection, programs and studies on autism, most low and middle income countries (LMICs) recently have started to classify and diagnose ASD. Increased cultural responsiveness and an enhanced understanding of ASD's scale and impact on society is the outcome of performing epidemiological research of autism. Although the writers of this study were unable to draw any firm assumptions or guidelines in these contexts on evidence-based procedures, they argued for the value of staff in primary and secondary health care clinical preparation and vital part of caretakers in the implementation of treatments. The writers made a strong call for researchers to fill the void in our expertise and apply current early intervention and technical preparation frameworks to low-resource conditions. As part of the 2007 Lancet series on the global impact of mental health problems, ASD is part of the WHO mental health portfolio, and researchers identified five key barriers to the growth of mental health services in LMIC:(a) shortage

of funding support and political commitment; (b) an over-centralized treatment system; (c) Lack of mental health care integration into the primary health system; (d) Lack of training for professionals; and (e) poor understanding of management in mental health. A concerted strategy involving the distribution of immediate, concrete benefits to people with ASD and their communities is required in order to achieve the aim of enhanced visibility, early identification and early action on a global scale, while continuing to promote larger structural improvements to allow lasting alternatives for the community.

2.4.1 Global Autism Public Health (GAPH) Initiative

Launched in 2008, the GAPH Project of Autism Speaks is an international scientific and education effort aimed at improving public policy strategies and successful autism awareness, study and service delivery initiatives. To date, partnerships have been set up with collaborators in more than 20 countries spanning six continents. There are several elements involved in the GAPH model. Firstly, by convening a National Advisory Committee (NAC), which is usually consisting of advocacy leaders, elected members, knowledgeable group members, international information consultants and Autism Speaks employees, local leadership is typically established. It is the responsibility of the NAC to define common group goals, to identify a range of programmatic opportunities and to create a strategic strategy, including taking into account the tools necessary for the execution of the plan. Second, an advocacy program has been initiated to identify the signs and effects of ASD and to minimize autism stigma and misconceptions.

Third, data related to the needs of the population is typically gathered to help prepare the public health system and services that would be required to benefit families and practitioners. This could include the design and delivery across current health centers of a national or provincial autism screening study or a full-scale incidence study performed by an autism epidemiologist. Fourthly, the development of resources for the provision of evidence-based health and educational facilities is parallel to the conduct of study. Successful therapies entail close cooperation with

clinicians and communities, and enable cultural backgrounds and beliefs to be receptive. Fifth, interventions that are given by the GAPH program, in addition to preservation, must be consistent with the sociopolitical sense of the society being served.

The NAC directs the development of a public health and regulatory system that is scientifically and financially viable, guided by the best available information, and will be responsive to the evolving needs of the ASD community by collaborating with communities, NGOs and government departments [52]. ASD, with major caregiver, family, and financial pressures, is one of the most severe neurodevelopmental cases in the United States.

It has been estimated that the annual cumulative ASD related costs in the United States are up to $250 billion, with lifetime actual ASD-associated costs in the range of $1.5 to $2.5 million (estimates in 2012 US dollars). Because of past underdiagnosis of ASD in older cohorts, these costs are undoubtedly underestimated, mainly because of this, one projection predicts that overall ASD-attributable costs would increase to over $450 billion by 2025 [53].

2.4.2 Impact of ASD on Families

At a time, children with ASD have multiple factorial and simultaneous conditions, their families tend to be more affected and need a wider variety of services. Additional financial pressure may be evident in their families with increased stress and mental health problems. Researcher found that there was a higher stress on families of autism patients who had no medical home than families who had a medical home. A health care system that is still not adequately prepared to meet the chronic care needs of ASD patients poses tremendous difficulties for children with complicated situations like autism.

Nonetheless, research explores the influence of ASD on family income, employment, and time. Families with elevated out-of-pocket expenses faced financial difficulties and reveals a demand for additional cash. For ASD patients at these

ages, the health insurance rate rises. Important changes have been made to expand coverage for people with ASD, but more diverse states and some large employee benefit plans had been implemented.

In order to provide for their child, parents of over half of children with special health care needs (CSHCN) with autistic patients had to decrease or stop work. Parents spend 10 hours a week with 25 percent of CSHCN with ASD organizing the treatment of their child's care [54].

2.5 Diagnosis of ASD

There are few systematic methods currently available to promote diagnosis of ASDs. Widely used are the Autism Screening Interview Revamped (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS).

2.5.1 The Developmental, Dimensional and Diagnostic Interview (3Di)

It is a comprehensive parent/caregiver screening assessment focused on computers and investigators in which individual symptoms will be scored from details given by parents and teachers of children with reported ASDs in terms of their seriousness and frequency. It comprises 740 modules, in which 183 evaluate history demographics, 266 modules estimate signs of autism patients, and 291 components explore probable comorbidity of other associative syndromes. Using an effective tool is important, to properly assess the severity, complexity and overlay of symptoms of ASD with other psychological disorders. Responses are recorded from '0' to '2' with no signs of impaired behaviour & definite evidence of such behaviour respectively. This diagnostic criteria can be utilized from early childhood through to maturity to diagnose people with ASD, and usually the test length varies from 1.5 to 2 h. ASD with the goal of strengthening the clinical treatment of patients with ASD. Evaluation methods include interviews with parents/caregivers, patient

interviews, direct patient evaluation, and comprehensive psychological evaluations. For ASD or other neurological disorders, this requires a rigorous study of family history. The following are among the scales which are commonly used to diagnosis ASD [38].

2.5.2 The Autism Diagnostic Interview-Revised

Autism Screening Interview-Revised (ADI-R) is an investigator-based interview of potential autism or ASD patients for caretakers of children. It encompasses 93 topics, including reciprocal social contact, communication or language, stereotyped repetitive habits and wishes, and age of inception parameters, which explicitly measure multiple behaviors at different ages. Usually, it is graded from '0' to '3' meaning no evidence and acute level of defective actions respectively. It needs around 2-3 hours to complete and can only be offered to those children who are 2 years old [38,55].

2.5.3 The Asperger Syndrome Diagnostic Interview

The Asperger Syndrome Diagnostic Interview (ASDI) is a concise 15 to 20 minute analyst based interview designed for the doctors to decide if a particular case of patient satisfies the autism or AS diagnostic criterion. It is made up of 20 objects split into 6 categories, i. Impairment of social interaction (four elements); ii. Interests limited (three elements); iii. Routines (Two elements) ; iv. Visual difficulties and expression problems (five elements); v. Non-verbal concerns of communicating (five elements); & vi. Clumsiness of motors (one element) [38].

2.5.4 The Diagnostic Interview for Social and Communication Disorders

A semi-structured interview for parents and guardians is the Screening Interview for Mental and Communication Disorders (DISCO) and is utilized in the diagnosis

of persons with autism from childhood to old age. It gives a developmental assessment of patient's social activity, abilities and speech from the early days to the present age. DISCO, which takes 2 to 3 hours to be delivered, uses a dimensional method for diagnosis rather than cut-off points, which seeks to closely examine the dynamics of impairments of social behaviors and communications of persons accused of developing signs of ASD over many years. It comprises of far more than 300 questions, but for diagnosis, only 93 are considers.

The digital code is used, for ranking i.e 0 = 'severe problem', 1 = 'minor problem' and 2 = 'no problem', since lower test scores show more signs. 38 products contribute to social contact impairments, 15 items refer to speech impairments, and 29 elements refer to stereotyped habits.

2.5.5 Autism Spectrum Disorder Diagnosis Scale for Intellectually Disabled Adults

To differentiate people with intellectual disability (ID) and ASD from adults with ID only, the Autism Spectrum Disorder-Diagnosis Scale for Intellectually Impaired Adults (ASD-DA) is used.

The ASD-DA contains 31 elements addressing three main deficits that can be administered in 10 minutes (social disability, communication deficits, and restricted interests), and usually graded as '0' for no impairment or '1' for impairment [38,55].

2.5.6 The Autism Spectrum Disorder Observation for Children

The ASD-OC is a 45-item observation scale which is used to observe and rate core autistic symptoms such as social dysfunction, coordination disorders, and repetitive behaviors. The behaviours of individuals with ASD are rated as '0' for no defect, '1' for moderate defect & '2' for severe defect. In children with age 3-15 years ASD-OC as an accurate scale for determining ASD symptoms [38]. The

ASD-OC assessment does not require professional preparation, but the clinician should at least have a clear understanding of the signs of ASD and familiarity of them.

2.5.7 The Autistic Behaviour Interview

The Autistic Behaviour Interview (ABI) is a standardized and diagnostic interview done by an expert with parents or caretakers. It contains 28 components representing a variety of acceptable and unacceptable activities, each components consists of 6 or elements & in total 168 elements are presents in an interview.

2.5.8 Behaviour Observation Scale for Autism

The Behaviour Observation Scale for Autism (BOS) is a direct indicator of observation for qualified clinicians and may be utilised on autistic children, mentally impaired children, and children that are normally growing. It is meant to differentiate between autistic children and children with other syndromes, such as learning disability, language deficiency and specific sensory deficits, in the following subgroups. 67 specified habits are included in the BOS, which are provided in checklist form and performed by an observer at nine, three-minute intervals. The activity event is measured as follows: 0 = The behaviour did not occur in the three-minute period. 1 = It occurred once. 2 = It occurred twice. 3 = The child engaged in the behaviour more or less constantly throughout the three-minute interval. Factor research has found that the majority of objects can be allocated to the following four categories: human interaction, object interaction, solitary actions, and stimulus response [55].

2.5.9 Childhood Autism Rating Scale (CARS)

The Childhood Autism Rating Scale (CARS) is a commonly used scale, widely used to help in diagnosis of autistic children. It helps to differentiate between

autistic children and children with other conditions of developmental dysfunction, that is intellectual disability. The effectiveness of CARS in diagnosing ASD in teenagers and adults has been confirmed by additional research. CARS comprises of 15 items covering multiple ASD signs and offers a reliable comparison of the activities and abilities of an infected child to a stable child's predicted developmental progress. Each object is graded from '1' to '4' including normal conduct to acute atypical behavior. Results between 30 and 37 show a mild to average ASD, and outcome between 38 and 60 specify acute ASD [38].

2.6 Medications / Pharmacological Therapies

Pharmacological and non-pharmacological behaviours are among the existing therapeutic strategies for ASD. Different forms of medicines, including psychiatric stimulants, NMDA receptor antagonists, abnormal antipsychotic medications, antidepressants, cholinesterase inhibitors, and anti-epileptic mood stabilisers, are used in pharmacological treatments [56].

Atypical risperidone, aripiprazole and antipsychotics are FDA drugs that are used to treat the associated symptoms of ASD but no specific drug for treating the core symptoms. However, some antidepressants, mood stabilizers, and other medicines are used for treating the acute conditions in ASD patients [57].

2.6.1 Psychostimulants

Since ASD and ADHD have a high degree of co-morbidity, treatment with conventional psycho stimulating ADHD medications as amphetamines and methylphenidate tends to be effective in the control of signs of ADHD in patients with ASD. Crossover RCTs, previously double-blind, indicated that methylphenidate could be useful for hyperactivity treatment in children with ASD.

It should be remembered that in ASD patients with little benefit from other signs such as irritability, social isolation, repeated activities, or speech dysfunction,

Psych stimulants were largely successful in refining co-morbid hyperactivity and impulsivity [38].

The most commonly used psych stimulant was methylphenidate, used in 80 percent of psych stimulant-treated cases, 52 percent dextroamphetamine, 20 percent mixed amphetamine salts, 17 percent pemoline, and 3 percent methamphetamine. In 66.1% of progenies with research-identified autism, psychopharmacologic drugs were included in this cohort. As stated in earlier research investigating the use of Psych stimulants in children with autism, methylphenidate was the most commonly used psych stimulant [58].

2.6.2 Atypical Antipsychotic Medications

Atypical antipsychotic drugs target variable-affinity subtypes of serotonin, dopamine and other receptors for neurotransmitters have been widely used to treat schizophrenia and other psychiatric conditions. Risperidone, Quetiapine, Aripiprazole, Ziprasidone and, to a lesser degree, Olanzapine are the atypical antipsychotic medications widely used by patients with ASD [38]. Clozapine is seldom used clinically as it can induce life-threatening agranulocytosis and involves fortnightly venipuncture to track white blood cell counts regularly on a weekly basis. Venipuncture is also not well accepted by cognitively disabled adolescents and adults with elevated degrees of irritability. Considering the lack of any known benefits of clozapine for these effects over other atypical antipsychotics, its use is restricted [59].

In the management with a number of signs of autism, atypical antipsychotics have been invaluable. They are also used for the management of irritability and related habits, including self-injury and hostility. For hyperactivity and stereotyped behaviour, they can also be successful. Medication-based therapies are also considered where therapeutic approaches are not entirely successful.

An analysis on psychotropic drug use in infants with PDD showed that a psychotropic drug is usually administered to only half of the participants and that 16.5”percent are taking an antipsychotic drug. Antipsychotics are most widely

used to treat mood and behavioural disorders that are marked by irritability, aggression, and agitation. The U.S. has officially accepted Risperidone. FDA for irritability therapy of infants and youth with autism. This approval is notable because it is the first licenced drug for use in autism and the first approved atypical antipsychotic for use in children and teenagers. Findings demonstrate that about 30% of PDD children and teenagers report mild to extreme irritability that is frequently accompanied by self-violence and other aggression.

2.6.2.1 Risperidone

Around 31 patients of mean age 28.1 years with autism and PDD NOS involved in the first placebo-controlled autism trial of Risperidone. It was a little bit more effective than placebo, with 8 of the 14 (57%) participants on the Clinical Global Impression-Improvement (CGI-I) scale being classified as responders against 0 of 16 in the placebo category. In specific, Risperidone has been successful in reducing interaction with repeat actions, as well as violence toward oneself, others, and assets. Significant variations were not captured between Risperidone and placebo on scales evaluating human and language social relations. At least one adverse effect was observed in 13 of 15 (87 percent) randomised to Risperidone subjects, but this included moderate, intermittent sedation in 5 subjects compared to 5 of 16 (31 percent) subjects given placebo. Weight gain resulted in a minority of subjects treated with Risperidone (2 out of 14) was observed. The Pediatric Psychopharmacology Research Units (RUPP) Autism Network conducted a double-blind, placebo-controlled trial of Risperidone in children and teenagers with autism [59]. A broader multicenter research performed by RUPP reported that Risperidone (mean dose 1.8 mg/kg) was given to 101 children and teenagers with ASD (5 to 17 years of age) for 8 weeks resulted in a 57 percent reduction in irritability vs. a 14 percent reduction in the placebo community. Treatment with Risperidone has increased anger, social withdrawal, repetitive activities, and hyperactivity dramatically. Research also suggests that low Risperidone doses (0.5-1.5 mg/d) in very young ASD children (aged 2-9 years)are active in reducing irritability and irritation [38].

2.6.2.2 Olanzapine

Olanzapine, an unusual agent, has been found to be effective in controlling a spectrum of schizophrenia, manic mania and treatment-resistant depressive symptoms. In addition, Olanzapine has been found to be effective in the controlling of a extensive variety of symptoms such as anxiety, violence, depression and suicide attempts [60]. In the management of aggressive activity in adult patients with schizophrenia, atypical antipsychotic drugs, Risperidone and Olanzapine, which inhibit postsynaptic serotonin and dopamine receptors, have been"found to be helpful and could be beneficial in adolescents with autistic conditions that have related behavioural disruptions. These Olanzapine trials included a small number of participants, ranging from 8 to 25 patients [61]. A double-blind RCT of 8 weeks confirmed that Olanzapine increased reaction rates in 11 young patients with ASD. Open-label therapy with Olanzapine (mean dose 7.8 mg/d) in 8 children, teenagers, and adults (age span, 5-42 years) substantially enhanced multiple symptoms such as irritability, anger, irritation, hyperactivity, social detachment and use of expression. During the 12-week experiment, the most influential adverse event was a rise in the"mean body weight of the participant by 8.4 kg[38].

2.6.2.3 Aripiprazole

The efficiency and care of Aripiprazole in ASD children is tested in two double-blind RCTs. In the first sample, 218 autistic children and adolescents (aged 6-17 years) randomized to receive either 3 separate doses of Aripiprazole (5, 10 or 15 mg/d) or placebo for 8 weeks were included. At 8 weeks of comparesion with placebo, all 3 doses greatly decreased irritability. Sedation, drooling, and tremor were the three most frequent side effects, This leads to a higher mean risk of discontinuation about 10.3 percent in patients treated with Aripiprazole vs. placebo which is 7.7 percent. The secondary RCT was an eight-week study of 98 children & teenagers who were diagnosed with autism and frequently exhibited Self-injurious or hostile behavior. A versatile dosage of Aripiprazole (mean dose of 8.6 mg/d) or placebo was given to the patients. As early as week 1, Aripiprazole

was successful in decreasing irritability and sustained its efficacy by the end of week 8 of the experiment. Aripiprazole group discontinuation rates were 10.6 percent vs. 5.9 percent in the placebo group. These findings culminated in Aripiprazole being accepted by the FDA as the second atypical antipsychotic medication to relieve irritability in children and teenagers aged 6-17 years of autism disorder.

2.6.2.4 Quetiapine

The findings of the Quetiapine clinical trials for the treatment of patients with ASD were inconclusive as most of these experiments were non-blinded or contained a retrospective chart analysis of the cases. Low response rates (25 percent) and high frequency of side effects includes sleep induction, agitation & weight gain were recorded in limited numbers of patients autism. In comparison, multiple case series trials observed greater response rates (about 40 to 60 percent) with severe side effects following treatment with Quetiapine in infants with ASD [38].

At first review, the reaction rate of Quetiapine in these unregulated trials is lower than that recorded for Risperidone. In both of these trials, the highest response rate was 60 percent. Somehow, relative to the other 3 trials, higher doses of Quetiapine were achieved. In order to determine more reliably its effectiveness and controlled trials of Quetiapine require an appropriate dosage in the treatment of autism and other PDDs[59].

2.6.2.5 Ziprasidone

The effectiveness, protection and tolerability of Ziprasidone in ASD children have been tested in two open label trials. Following 14 weeks of Ziprasidone therapy (mean dose of 59 mg/d), one study reported a substantial increase in aggression, irritation, and irritability. The drug was well accepted and also lost weight in a few patients (mean weight lost 5.8 lb), which could be due to transitioning from other atypical drugs typically associated with severe weight gain. Another research tested the results of transitioning to Ziprasidone in 10 autistic patients

who experienced significant weight gain during taking Risperidone or Quetiapine. To validate ziprasidone therapeutic effects in ASD patients, additional double-blind RCTs are required.

2.6.3 Antidepressants Drugs

In adults and infants, antidepressants, specifically selective serotonin reuptake inhibitors (SSRIs), are the fourth most widely prescribed and third most commonly prescribed psychotropic medications. SSRIs are potentially effective medications for the prevention of anxiety, repeated habits, or central ASD symptoms. There is currently very little evidence on antidepressant effectiveness in patients with ASD and data on SSRIs are varied and inconsistent.

For example, in children aged 5-17 years, the largest documented study of citalopram showed little impact at all on repeated and compulsive activity, but indicated a potential advantage on demanding behaviour. Additionally, the large placebo-controlled 'Study of Fluoxetine in Autism' (SOFIA) study found that fluoxetine was not successful as opposed to placebo in minimizing repeated activities"in children and adolescents with ASD.

Evidence has not proven that SSRIs have been successful in treating ASD in infants, and these medications may cause harm, while some positive effects has been manifested in autism adults with less side effects including headache, vomiting and sleep induction.

Moreover, a relatively limited double-blind, placebo-controlled adult fluoxetine study showed promising outcomes for repeated habits and global severity, but no change in other effects.

2.6.3.1 Sertraline

Few tests in adults with ASD have tested the effectiveness and tolerability of sertraline. A small open-label clinical study showed that in 8 out of 9 paediatric

autistic subjects, 2-8 weeks of treatment with sertraline (25-50 mg) significantly improved anxiety and irritation. In a wider open-label trial, 12-week treatment with higher doses of sertraline was found (50-200 mg) in adult ASD patients decreased violent and repetitive behaviours. With few adverse events, the drug was well tolerated. To determine the effects of sertraline in ASD patients more extensively, double-blind RCTs are also required.

2.6.3.2 Citalopram

Citalopram has been documented in a retrospective open-label trial to enhance agitation and hostility in little amount of ASD children, without modifying the main symptoms of disorder. Another observational study analyzing the medical charts of 15 young ASD patients showed that with moderate side effects, citalopram decreased agitation, repeated habits, and irritability.

2.6.3.3 Escitalopram

There are scarce trials testing escitalopram in ASD. In a 10-week open-label trial, escitalopram increased impulsivity and general psychosocial functioning relative to placebo in young ASD patients. However, a significant percentage of patients reported side effects that contributed to early cessation of therapy, such as hyperactivity, agitation or irritability. The treatment of escitalopram in adult ASD patients with and without comorbid depression or anxiety requires additional double-blind RCTs.

2.6.3.4 Fluvoxamine

Clinical outcomes for ASD with fluvoxamine are also indecisive. An open-label research showed that 83% (15 out of 18) of adolescent ASD patients with comorbid anxiety and compulsive symptoms did not display a substantial difference in their symptoms of ASD or anxiety after 10 weeks of low-dose (1.5 mg/kg/day) fluvoxamine therapy. These patients have reported a high rate of adverse events such as

akathisia, anxiety, headaches, sleep difficulties and changes in appetite. In comparison, a 12-week double-blind RCT indicated that fluvoxamine may have some therapeutic advantages relative to placebo-treated patients in terms of minimising repeated thinking, attitudes and violence in adult ASD patients.

2.6.4 Alpha-2 Adrenergic Receptor Agonists

The usage of adrenergic receptor agonists for alpha-2 is consistent with the handling of patients with ASD with aggressive activity, sleep disruptions and anxiety, which are prominent signs.

These drugs block neurotransmission of norepinephrine in the brain stem, leading to a reduction in sympathetic outflow and peripheral resistance, thus decreasing hyper-arousal, agitation, and/or muscle spasm states.

A prior double-blind, crossover RCT documented that in young ASD patients, 4-week therapy with the alpha-2 adrenergic receptor agonist clonidine decreased hyper arousal activities and increased social experiences.

Since treatment with clonidine, another double-blind RCT demonstrated slight enhancement in irritability and hyperactivity in offspring with ASD.

A third open-label observational clinical trial showed that clonidine was successful in minimising the delay of sleep instigation and night waking and, to a limited extent, in enhancing hypertension and aggression in children with ASD, with a relatively mild tolerability profile [38].

2.7 Management Strategies /Non-Pharmacological Therapies for ASD

Limited treatment options are available to cure the core symptoms of ASD. Psychosocial therapies help to target some core and associated symptoms of ASD.

2.7.1 Applied Behavior Analysis (ABA)

Applied behavior analysis (ABA) is an option of treatments which is based on the theory of learning and operant conditioning. It includes the use of positive reinforcement (praising, edible rewards etc.). A meta-analysis shows positive effects on language development, social and intellectual functioning, but its time taking process [62].

ABA interventions targets behavior, and implement structure specific teaching methods which includes language, cognitive sensorimotor skills, social interactions and specific behavior problems. ABA includes Early Intensive Behavioral Intervention (EIBI) for children under 5 years worked by decomposing complex skills into elementary subskills as well as teaching them individually. It increases intellectual functioning in 50% of yound ASD children. Other ABA programs include the Learning Experiences An Alternative Program for Preschoolers and Parents (LEAP), which focuses on integrated teaching with non ASD peers, and emphasizes individual-based curricula [38].

2.7.2 Pivotal Response Treatment (PRT)

Another treatment strategy used is Pivotal response treatment (PRT), it targets the naturalistic behavior patterns such as specific skills that are vital to development across social, communication, behavior and motivations. Based on ABA principles, pivotal further refers to set of targeted skills which when successfully obtained, can bring out some positive clinical gains in other domains of functions in the patients In contrats to traditional ABA, it triggers correct behavioral responces and valid attempts for skills accession in order to increase childs motivations [63].

2.7.3 Cognitive Behavior Therapy (CBT)

In the treatment of co-morbid anxiety in children with ASD, cognitive behavior therapy (CBT) is used. Researchers have presented proof of the success of CBT

interventions for school-age and young ASD adolescents. Improvements have been identified in anxiety, self-help, and everyday living skills, with 78 percent of 7-11 year-olds in the CBT-treated community classified in one study as supportive responders [62]. However, 24 studies have tested the efficacy of CBT for ASD symptoms. Both the effect of CBT on social skills and anxiety were also explored in the studies [64]. The highly coordinated and highly predictable nature of CBT makes it particularly ideal for patients with ASD to target both core symptoms and comorbid anxiety and depression [38].

2.7.4 Social Behavior Therapy (SBT)

Social behavioral therapy focuses on emotional control, social skills and communication development. SBT techniques include both simple tailored strategies as well as more nuanced strategies.

SBT also provides models of developmental intervention, in which a systematic assessment based on developmental history, function evaluation, and clinical findings of experiences is carried out to provide a developmental profile for each patient, classify affected domains, and design tailored interventions.

The models include the Denver Model and the Receptive Teaching of the Early Start Denver Model (for toddlers) (ESDM) and the model based on the Developmental Individual-Difference Relationship (DIR).

2.7.5 Herbal Medicine

In patients with ASD, various herbal remedies such as Gingko biloba, Zingiber officinale (ginger), Astralagus Membranecaeus, Centella asiatica (gotu cola), and Acorus Calamus (Calamus) may have therapeutic benefits, including increased cerebral circulation, enhanced cognitive function, calming or sedative effects, and improved immune response. Herbal medicines showed positive results in improving abnormal habits and inattention in ASD patients when used in conjunction

with traditional therapy. For the validation of possible therapeutic benefits of herbal remedies in ASD, additional randomized placebo-controlled trials (RCTs) are required [38].

2.8 Neurobiology of Autism Spectrum Disorder

Molecular and cellular abnormalities of early brain development is seen in ASD. In order to explain the development of autism symptoms, intercellular connectivity, an imbalance between some neuronal populations and the maturation/differentiation phase, oxidative stress induced by embryonic stages, could be promising candidates.

The prevalence rate of autism was reported to be 1 percent or 2 percent in multiple studies. In order to get a better understanding of these processes, valid "disease models" are important. A modern cutting-edge technique, called brain organoids, was highlighted as a potential candidate to achieve a better "disease model."

In vivo timeline development is followed by brain organoids generated from patients induced by pluripotent stem cells (iPSC) they also have the capacity to recreate the proper brain complexity, developmental stages.

At the level of cellular and gene expression, organoids display a clear similarity to the developing brain in vivo and can therefore recapitulate the early phases of neurogenesis.

Organoids are the most important in vitro cellular platform currently available to explain the pathways behind the ADS pathology. Mini-brain research will offer a larger and more detailed description of the complex disorder and thereby, at various points in its development, establish therapeutic and preventive strategies.

It is a tool that can be used as a potential drug for a successful high-throughput screening of chemical compounds (in sphero drug test). Organoids are a good modelling system to describe the role of epigenetic and environmental factors in the development of ASD [65].

2.8.1 Amygdala and Autism Spectrum Disorder

Amygdala is a major component of the social cognition neural network. Amygdala, which is mainly associated in the brain region with emotional processes. Due to the structure's almond like shape, amygdala is derived from the Greek word amygdale, meaning "almond." It is situated in the temporal medial lobe, just before the hippocampus (in front of it). It is a paired structure similar to the hippocampus, with one situated in each brain hemisphere. The amygdala is the part of the limbic system in which a neural network that mediates multiple aspects of emotion and memory. Although the amygdala has traditionally been thought to be predominantly involved in fear and other feelings associated with aversive (unpleasant) stimuli, it is now understood to be involved in positive emotions elicited by appetitive (rewarding) stimuli. A collection of nuclei, or clusters of neurons, form the amygdala. A function associated with the amygdala ASD is characterized by impairments in"social cognition. Subdivisions of the amygdala have been established that demonstrate structure, connectivity, and function specificity. Within ASD, nothing is known about amygdala connectivity. The purpose of this study was to investigate the microstructural properties of amygdala-cortical links and their connection with ASD behaviors, and whether specific amygdala subregion connectivity is correlated with specific ASD characteristics. This correlation between the amygdala and emotional intelligence development indicates that structural and/or functional defects in the amygdala, particularly during childhood, may play a key role in the development and maintenance of autistic behaviors, a term known as the 'autism theory of amygdala.' No study has examined amygdala connectivity specifically in ASD, despite evidence from lesion studies and volumetric tests highlighting the role of the amygdala in ASD, to the best of our knowledge. Microstructural variations in WM pathways that bind to temporal lobe structures have been explored in a variety of previous ASD studies in young children with ASD. Evidence indicates that in ASD, sub regions of the amygdala are cytoarchitecturally altered, with increased cell packing density and decreased neuronal size found in medial amygdala nuclei in particular. Contrary to this, in ASD, decreased neuronal density has been recorded in the lateral amygdala nucleus [66].

2.8.2 Prefrontal Cortex and Autism

A large number of recent studies have demonstrated evidence of impaired executive functions in autism spectrum disorder, together with well-documented disturbances of social interaction, communication, perception and focus. Although the frontal lobes have long been recognised, especially the prefrontal cortex, in higher-level control as playing an important role. Neuroimaging and neuropsychological research have only recently started to delineate distinct prefrontal cortex regions promoting different aspects of executive function. In part, the evidence for such differences emerged from studies of patients with frontal lobe lesions who encountered behavioural disorganization with such intensity in daily life that they were unable to return to work at their previous level, but performed well on classical executive function measures such as the Stroop challenge, Wisconsin Card Sorting Test, Tower of London, verbal fluency, etc. The prefrontal cortex, located just behind the forehead, may show some structural abnormalities in individuals with autism. A postmortem study shows that children with autism in the prefrontal cortex have more neurons than controls do.

They can also contain patches of immature cells which do not exhibit the cortex's characteristic layered organization. Autistic people's imaging tests also reveal atypical functional relations between the prefrontal cortex and other regions of the brain. Several studies, for instance, have reported unusually poor long-range links, and one study found that individuals with certain CNTNAP2 gene mutations display increased connectivity within the region. Other autism genes can also influence the structure and function of the prefrontal cortex, as they are expressed there at high levels during mid-fetal development, a critical time for the risk of autism. A hotspot for the involvement of the immune system in autism also tends to be the prefrontal cortex. Therefore,a research was performed to compare the responsivity of the prefrontal cortex functional communication in adults with and without ASD.

The prefrontal cortex was therefore chosen as a region of interest (ROI), since this region is reliably involved in the core symptoms of ASD, including deficits in mind

theory and socio-emotional response control [67].

2.8.3 Nucleus Accumbens and Autism

The NAc is regarded as a key structure in ASD relevant to social reward response. The nucleus accumbens (NAc) and basolateral amygdala are part of the circuit involved in expression of autism and associated behavioral symptoms. Deep brain stimulation (DBS) of these structures has been studied with minimal success in 4 cases of ASD1 in amygdala, 2 cases in globus pallidus internus, and 1 case in NAc.

Obsessive compulsive disorder (OCD) and aggression have also been controlled using NAc DBS. To control her anger and OCD, we gave NAc DBS to a woman with autism and were delighted to see changes in autistic symptoms. In order to explain the dynamic function of NAc in autism, we present this case as an example. NAc, is the central hub that receives important afferents, like amygdala, the orbitofrontal cortex (OFC), anterior cingulate cortex (ACC), and medial temporal lobe. Such findings make NAc a possible target for modulation in ASD. NAc shell high-frequency stimulation (HFS) has also raised NAc dopamine and serotonin concentrations, which are shown to be responsible for symptom reduction. Preliminary evidence suggests that in individuals with ASD, the motivational component of reward (the desired component) may be especially compromised. This shows that VS and NAc in patients with ASD are main structures in the dysfunctional circuit [68].

2.9 Comorbid Disorders

In simple terms, comorbidity refers to the presence of more than one disorder in the same person. For example, if a person is diagnosed with both social anxiety disorder (SAD) and major depressive disorder (MDD), they are said to have comorbid (meaning co-existing) anxiety and depressive disorders. The term comorbidity was coined in the 1970s by A.R. Feinstein, a renowned American doctor,

and epidemiologist. Feinstein demonstrated comorbidity through the example of how people who suffered from rheumatic fever also usually suffered from multiple other diseases. Since that time, comorbidity has come to be associated with the presence of multiple mental or physical illnesses in the same person. It's not uncommon for people to suffer from two disorders or illnesses at once.

Comorbidity in mental illness can include a situation where a person receives a medical diagnosis that is followed by the diagnosis of a mental disorder (or vice versa), or it can involve the diagnosis of a mental disorder that is followed by the diagnosis of another mental disorder. The high rate of comorbidity between substance use disorders and other mental illnesses calls for a comprehensive approach that identifies and evaluates both.

Accordingly, anyone seeking help for substance use, misuse, or addiction or another mental disorder should be evaluated for both and treated accordingly. For the treatment of comorbid disorders, some behavioral treatments have shown promise. These interventions may be personalized to patients based on age, misuse of specific medications,and other variables. They can be used individually or in drug combinations [69].

2.9.1 ADHD

Attention Deficit hyperactivity disorder (ADHD) is a disorder of the brain that affects attention and control of an individual's behavior. The ratio of co-morbidity between ASD and ADHD is very high. Evidence shows that about 30 to 50 percent of ASD patients also show symptoms of ADHD, and 66 percent of ADHD patients also show ASD characteristics [70]. In children, the most widely diagnosed psychiatric disorder is ADHD, as ASD also affects boys more than girls [38].

During the early school years, it is usually visible when a child appears to have trouble paying attention. ADHD can not have specific preventions or cures, but a child can be helped to manage his symptoms with early and good treatment and education plan. It affects those areas of the brain that control attention. Poor

nutrition during pregnancy, infections, smoking, drinking, and substance abuse. These factors can affect a baby's brain growth. Damage to the front of the brain may cause problems with impulse and emotion regulation, called the frontal lobe [71].

2.9.2 Depression

Depression is a prevalent mental disorder that affects more than 264 million people around the world. It is characterized by recurrent depression and a lack of interest or pleasure in previously rewarding or enjoyable activities. It is the leading cause of disability in the world and significantly contributes to the global burden of disease [72].

It often occurs in ASD, although in many cases it is hard to get a correct diagnosis of depression in autistic patients and dua to the masking of depressive symptoms in autism it remains untreated [73]. Prevalence thresholds for depression ranges from 10 percent-50 percent among young ASD patients. Findings from the database of the Interactive Autism Network suggest that 11 percent of ASD depression has also been diagnosed in children [38]. Depressive episodes in patients with ASD achieve more symptomatic severity and last longer than in patients of ASD children with either neuropsychiatric disorders of non-ASD or healthy development [74].

2.9.3 Anxiety Disorders

Anxiety disorders are a category characterized by significant feelings of anxiety and fear that are the most common mental health problem. Anxiety is a worry about future events, while fear is a response to current events. There are several anxiety disorders, including generalized anxiety disorder, particular phobia, social anxiety disorder, anxiety disorder of separation, agoraphobia, panic disorder, and selective autism. Anxiety disorders are the most common subgroup of mental illnesses [75]. One or more anxiety illnesses affect up to 80 percent of children with ASD as well. Separation anxiety disorder (SAD) has the largest incidence of ASD

comorbidity (38%), about 37% of obsessive-compulsive disorder (OCD), 35% of generalized anxiety disorder (GAD) and 30% social phobia (SP). The involvement of AD normally compounds functional psychosocial disorder and raises the severity of symptoms of ASD [38]. Anxiety comorbidity in children and adolescents with ASDs has been extensively studied. Around 54 adults with high functioning ASD were affected by at least 1 anxiety disorder in the current sample, 56 percent of them, while about 20 percent had 2 or more anxiety disorders [76].

2.9.4 Bipolar Disorder

Bipolar disorder is a mental health condition that causes extreme mood changes, including emotional and low mood swings, previously called bipolar depression (depression). Bipolar disorder patients may have moments when, in other cycles, they feel extremely enthusiastic and energized and feel very sad, powerless,and low. They usually feel normal in between those times [77]. In ASD patients, this disorder is recurring and occurs during puberty.

A research showed that 30 percent of patients with BPD have also consulted with ASD requirements and that concurrency led to the onset of BPD symptoms at an earlier age. Similarly, 44 young, highly functioning ASD patients documented a high BPD co-morbidity prevalence in another study [38]. Some research indicates that a substantial number of patients with ASD have bipolar disorder [78].

2.9.5 Tourette Syndrome (TS)

Tourette syndrome is a neurological disorder characterized by repetitive, stereotyped, involuntary gestures and speech patterns called tics (TS). Usually, early signs of TS are noticed as ASD in infancy, with an estimated emergence between the ages of 3 and 9 years. Research shows that anomalies in some regions of the brain (including the basal ganglia, frontal lobe, and cortex), the interconnecting circuits of these regions, and the neurotransmitters (dopamine, serotonin, and norepinephrine) are responsible for nerve cell communication [79]. Several studies

indicate that TS co-occurs more often than in the general population in patients with ASD. One research recorded that comorbid PDD was also present in 4.6 percent of TS patients. Another research shows that signs of TS and other recurrent motor tics occur in about 22 percent of ASD patients. The higher prevalence of ASD with TS suggests that common genetic features or associated synaptic neurotransmission imbalances could be shared by both conditions [38].

2.10 Signaling Pathways Involved in Autism

ASD is a group of heterogenous and multifactorial disorder, representing severe burden on patients. For understanding the multifactorial functions of ASD, signalling pathways have been targeted to know the crosstalks of the pathways. Many experimental platforms were used to identify diverse mutations in the core components of the several many cell signaling pathways.

These signaling cascades control a wide range of neurological features such as neuronal development, neurotransmission, metabolism, and homeostasis, as well as immune regulation and inflammation. Moreover,the signaling pathways involved in ASD had been highlighted which includes, developmental pathways (Wnt, Sonic hedgehog, Retinoic acid), metabolic pathways(Mapk/Erk, P13k/Akt), proinflammatory and cytokines pathways(TGF-beta, Jak-Stat, NF-kb) [11].

2.10.1 Developmental Pathways Affected in ASD

The meaning of 'development' has been variously defined, but various definitions converge to the point that throw light on the systematic and structured process involving changes in character that are successive and take place over one's lifetime. Thus, changes in structures as well functions are known to be developmental changes. Development also involve differentiation and structuring of previously unstructured fields [80]. The human brain undergoes many developmental changes, which causes complex cognitive abilities, these includes the language, reasoning

and cognitive controls. These abilities are made possible by trajectories of brain development [81].

2.10.1.1 Altered Wnt/Beta-Catenin Signalling Pathway

Wnt is the combination of two words driving from Drosophila segment polarity gene wingless and name of integrated vertebrate homolog (INT) [82]. Human INT1 shows similarity with mouse INT1 genes, hence manifesting its conserved nature across various species [83]. Mutation in Wnt may lead to human birth defects, cancer, and other related diseases [84]. Wnt protein family contains 19 secreted glycoproteins highly preserved in species, from invertebrates to mammals [83]. Wnt includes both canonical and non-canonical pathways. Non-canonical or beta-catenin independent pathways can be further divided into the planar cell polarity pathway and Wnt/Ca2+ pathways. Wnt binds to frizzled G- protein, a seven-transmembrane receptor, and LRP5/6 a low-density lipoprotein act as co-receptor of Wnt ligand [85]. Activation of FZD transmits a message into the cytoplasm and dissociation of the cadherin complex occurs. Beta-catenin is determined by the destruction complex including two serine-threonine kinases(CK1 alpha and GSK3) two scaffolding proteins(axin and APC)and phosphatases PP1 and PP2A [86]. In ASD the canonical pathway is increased while peroxisome proliferator receptor gamma (PPAR gamma) and PGC-1 alpha are decreased which acts as coactivators and belongs to the superfamily of ligand-activated transcriptional factors act in the integrity of the central nervous system. Dysregulation of PPAR gamma leads to dysfunction in CNS. It also decreases the effect of NF-kB to decrease inflammation. Hence, showing a cross-talk between them [87]. Dvl and GSK-3 beta inhibitors have been shown in cognitive and behavioral abnormalities. PRICKLE2, ANK3, UBE3A, DIXDC1, PGE2, TBL1X, Wnt1,Wnt3, Wnt9B, Dyrk1A, Shank3 and NLGN3 genes are also contributing to ASD by disturbing Wnt canonical and non-canonical pathways [88,89].

In ASD Wnt2 occurs in the hotspot region of chromosome 7q31 and is a strong candidate gene involved in autism [86,11]. Wnt2 acts as an antibody for FZD9 so

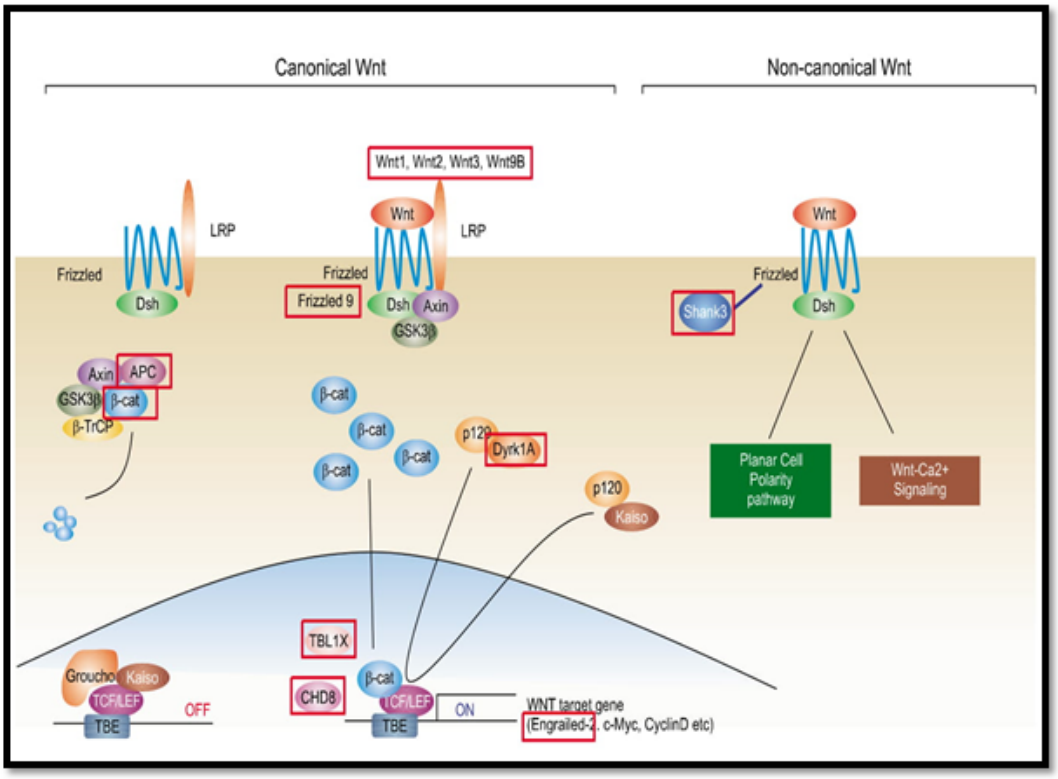

FIGURE 2.2: Canonical and non-canonical pathway regulation. Beta catenin is degraded by destruction complex when no ligand is attached to receptor. By association of Wnt ligand with receptors (Frizzled and LRP5/6) beta catenin becomes stabilized, it enters the nucleus and activates Wnt target genes such as Engralied-2. The red boxes shows the mutated genes of ASD, which are identified from NGS [47].

act as a preferred receptor, its mutation loss of function, and duplication could be seen in ASD patients. BCL9 present on 1q21 promotes the transcription activity, duplication of this area may lead to autism [86]. Chromodomain- helicase-DNA-binding protein (CHD8) function as an ATP-dependent chromatin remodelling factor, it serves as Wnt's negative regulator. It recruits histone H1 to the target gene, the mutation in CHD8 causes ASD [90,91]. Neuroligin 3 genes (NLGN3) co-incides with 50 high ranking genes in SFARI genes [92]. CTNNB1 itself is involved in severe mental disabilities which are also identified in ASD patients through exon sequencing [93]. Other genes include APC, DISC1, EN2, MET, WIF1, MARK1, CDH10, WNT2, PTPRZ1, CDH15, CDH13, CDH8, DOCK4, BCL9, FZD9, AHI1, CREBBP, TSC1/2 [86] CHD8, ARID1B, DYRK1A, SYNGAP1, ADNP, ANK2, DSCAM, SCN2A, ASH1L, CHD2, KDM5B, and POGZ genes are present in ASD

patients. While CHD8, ARID1B, DYRK1A, SYNGAP1, ADNP, ANK2, DSCAM, SCN2A, ASH1L, CHD2, KDM5B, and POGZ genes are considered to be high confidence risk genes [94,89]. Disheveled is phosphorylated by MARK-1, the operation of the APC/Axin/GSK-3β complex is suppressed. Overexpression and genetic silencing of MARK-1 resulted in too small dendrites being a key finding. The Wnt pathway therefore plays a key role in autism pathogenesis [95].

2.10.1.2 Sonic Hedgehog (SHH)

Sonic hedgehog is a Hedgehog family-related signalling protein. SHH interacts with its Patched-1 (PTCH1) receptor that cannot associate with the smoothened (SMO) trans membrane protein. This affiliation has contributed to activation of the GLI transcription factor. In a wide variety of cells, GLI regulates the expression of different target genes; including neurons, which regulates cell growth, differentiation, and survival. During embryonic development, it is known to be important. Prior studies have been shown that SHH signalling is involved in the tissues repair process in the adult organism and is regulated after injury. In vertebrate brain, SHH has a fundamental role [11]. Shh causes neurotrophic and neuroprotective effects on neurons of CNS. Downregulation of neurotrophic levels originating from B-cells lymphoma 2 (Bcl-2) and the brain, such as glutathione depletion, lower peroxide production and glutathione peroxidase, mediates apoptosis and oxidative stress in autism [96]. The oxidative stress arising from reactive species of oxygen (ROS) excess generation is well identified. Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and ASD, ROS are a principle contributing factor. SHH signalling activation results in defence of cortical neurons against oxidative stress [97].

BDNF is a small protein present in the central nervous system and peripheral blood. It is a widely distributed neurotrophin within CNS. In ASD, oxidative stress might be associated with BDNF. A decreased in BDNF in ASD children was sen in the previous studies [98]. Higher serum levels of SHH and BDNF were seen in autistic patients, indicating their pathophysiological aspect in ASD

[96,99]. 7-dehydrocholesterol reductase (encoded by DHCR7) is malfunctioning in patients with this condition and thus results in affected cholesterol biosynthesis and physical disorders such as microcephaly, syndactyly, heart defects, mental retardation and autism characteristics [89,11]. A transcription factor Engrailed2 (EN2), has been ASD linked. Cholesterol a structural unit of myelin acts as a substrate for normal neural function and facilitates SHH. Its dysfunction also leads to ASD by impaired SHH pattering, abnormal synaptic plasticity and alteration in membrane lipids during brain development [96].

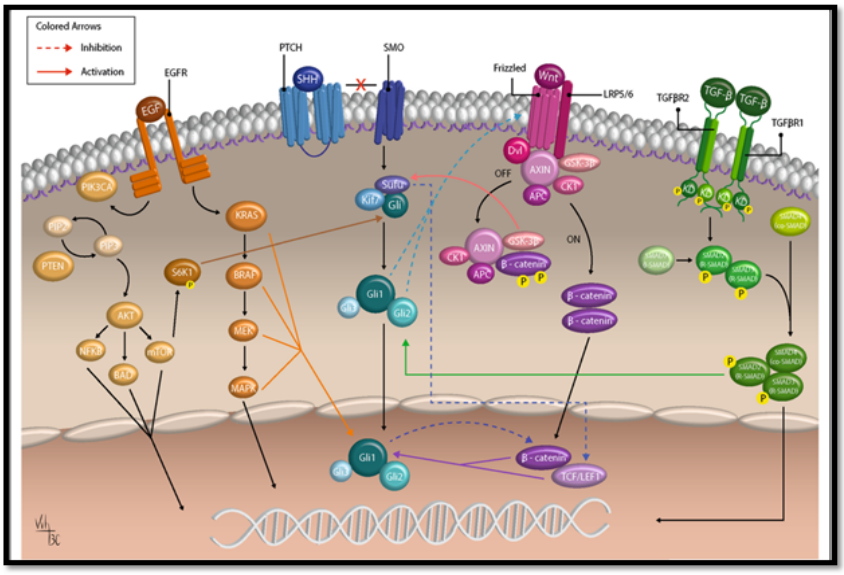

FIGURE 2.3: The crosstalk among SHH and other pathways. It includes Wnt (purple), TGF- beta (green). At different times, there is crosstalk among them, it is more important to understand molecular interaction in order to look for more therapeutic targets [100].

2.10.1.3 Retinoic Acid Signaling

Retinoic acid (RA) can influence various developmental genes in their regulatory regions containing RA response elements (RARE). It is not unusual that interconnections between RA and neurodevelopmental disorder, such as autism, they were discovered due to the role of RA as a developmental operator. It controls a group of early homeobox (HOX) genes during embryogenesis. The pattern of the anterior posterior upper body, including patterns of brians, is formed by these HOX

genes. In the differentiation of many types of neural cells, including GABAergic and dopaminergic neurons, RA is involved. Retinol deficiency in pregnant rat reduces the hypothalamus production of RA receptors (RAR, beta isoform), contributing to increased offspring autistic like behaviour. In vitor, an increased degradation rate of ALDH isoform (ALDH1A2) due to ubiquitin ligase E3, hyper ubiquitinoylation was reported and in mice overexpressing UBE3AA, autistic activity was observed. Adversely, influencing neurodevelopmental gene expression such as HTR2C, CHD7, CDH8, CDH9, EPHA7, PCDH20 and SEMA3A3A3A in certain autistic patients, mutations in RAR/ RXR gene co-activator complex (RERE) have been identified, suggesting the need for more studies into RERE genetic modifications linked to autism pathogenesis [11].

The deficiency of vitamin A can induce behaviors similar to ASD in rats. In a subset of autistic patients, a low level of ALDH1A1 was found. UBE3A overexpression represses ALDH1A2 and impairs ASD synaptic plasticity mediated by RA, which can be miligated by RA supplements [89]. To sum up, CD38 is a multifunctional molecule that combines enzyme and receptor properties and plays a key role in various tissue processes. In brain's neurons and glial cells, CD38 is present. The gene expression and family-based relationship included the SNP (rs3796863), which was significantly associated with ASD [101]. The UBE3A gene, an E3 Ub ligase, was reported as the key contributor to 15q11-q13 CNV symptoms in 1-3% of ASD cases worldwide, maternal replication of it and isodicentric chromosome 15 in several population based studies, three main autism behavioural traits were also seen when transgenic mice displaying UBE3A at high dosage, thereby defining UBE3A hyperactivities as one of best known genetic triggers of subtypes of ASD [102]. In our previous inquiry, 7.9% introverted children were found to have vitamin A insufficiency. In children with ASD, serum retinol levels have been significantly increased and serum 5-HT levels have been reduced [103]. It is recognised that more than 1000 characteristic are included in ASD, most of them contained in characteristics of know work and included in pathways of cell and neuronal formation, projection, motility and expansion also in characteristics included in spine and dendrite control flexibility and quality control [104].

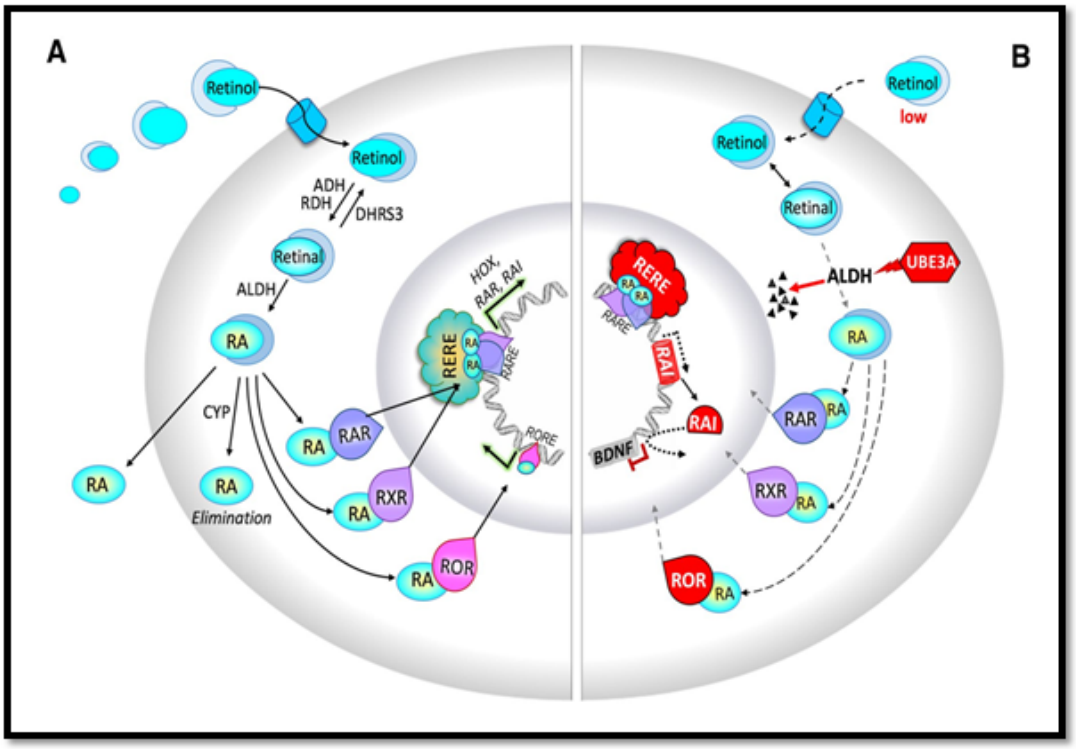

FIGURE 2.4: The pathway of the RAS-RAF-MEK-ERK. A small G protein (RAS) and three protein kinases (RAF, MEK, ERK) are included in the pathway's general structure. (A kinase is an enzyme that catalyzes the transfer of a phosphate group from a donor molecule to an acceptor). The binding of the ligand to a transmembrane protein, receptor tyrosine kinase (RTK), is the starting point for this pathway. The resulting signalling cascade corresponds with the translocation of ERK (MAPK) to the nucleus, where ERK stimulates transcription factors that result in gene expression. The initial point is the binding of a ligand to a transmembrane protein, the extracellular portion of the two receptor tyrosine kinases (RTK) subunits (e.g., growth factor, cytokine, or hormone). Ligand binding allows a dimer to form the subunits and leads to their cytoplasmic domains being phosphorylated. This RTK activation causes cytoplasmic adaptor proteins to bind to it (not shown). In comparison, guanine nucleotide exchange factors (GEFs) are drawn to the plasma membrane by the adaptor proteins, where a small G protein-like RAS is activated. RAS usually is in its inactive state, binding guanosine diphosphate (GDP) [66].

2.10.2 Metabolism Pathways in ASD

Metabolic pathways and cycles are several response chains in which the next stage of the process becomes the substrate for chemical products. These substrate in the reactions either belongs to pathways if the reactions are linear or contributes in

metabolism cycle. All process substrates are chemically modified in the reactions of signaling pathways [105].

2.10.2.1 MAPK/ERK Signaling Pathway

The main signaling pathways involved in controlling the proliferation, survival, and differentiation of normal cells are mitogen-activated protein kinase (MAPK) cascades. Importantly, MAPKs shown involvment in certain genetic and psychiatric disorders, particularly the ERK / MAPK pathway. In a collection of syndromes called neuro-cardio-facial-cutaneous syndromes (NCFC) or Rasopathies, mutations in genes encoding various components or regulators of the ERK / MAPK pathway have been identified. ASD have recently been recognised to be associated with these disorders. A central hub that interacts with many of the genes and CNVs that have been involved in ASD is the ERK/MAPK pathway. Several syndromic forms of ASD have been established by the body of literature, with widespread dysregulation in the ERK / MAPK pathway, and these disorders are now generally referred to as "Rasopathies" Ras, which activates the ERKs, because of mutations in genes encoding elements of this signalling pathway located upstream and downstream of the G-protein [106]. In idiopathic ASD patients, studies of genome-wide association (GWAS) have shown that the ERK / MAPK pathway is a convergence site for many of the known genes. In addition, In 2446 ASD subjects and their relatives, a GWAS review of CNVs and SNPs recognized the MAPK signaling pathway as a general point of convergence for many pathogenic CNVs and single nucleotide variants (SNVs) [106,108]. The two hub genes strongly associated with ASD network pathways were considered to be the MAPK1 (ERK2) and MAPK3 (ERK1) in order to classify the most relevant genes for the disorder. In people with cardiofaciocutaneous syndrome, an abnormal genetic disorder caused by mutations in KRAS, BRAF, MEK1 and MEK2, autistic symptoms are also observed. Costello Syndrome (similar to ASD), a complex developmental disorder characterized by delayed physical growth and learning delays, is often caused by multiple HRASS mutations [11]. In patients with syndromic ASD, the MAPK pathway is unregulated. Also, the 16p11.2 locus

deletion, which involves the gene MAPK3 (mitogen-activated protein kinase 3), is associated with ASD [109].

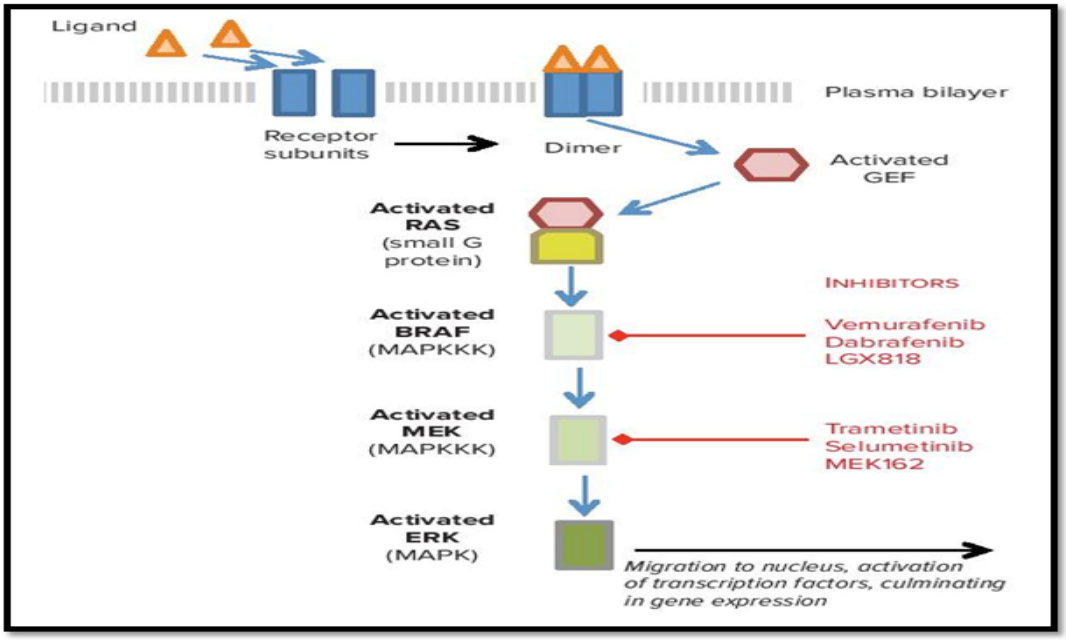

FIGURE 2.5: The pathway of the RAS-RAF-MEK-ERK. A small G protein (RAS) and three protein kinases (RAF, MEK, ERK) are included in the pathway's general structure. The binding of the ligand to a transmembrane protein, receptor tyrosine kinase (RTK), is the starting point for this pathway. The resulting signalling cascade corresponds with the translocation of ERK (MAPK) to the nucleus, where ERK stimulates transcription factors that result in gene expression. In comparison, guanine nucleotide exchange factors (GEFs) are drawn to the plasma membrane by the adaptor proteins, where a small G protein-like RAS is activated. RAS usually is in its inactive state. When GEF displaces GDP from the RAF and allows GTP to bind, RAS becomes transiently active; RAS then cleaves the linked GTP and becomes inactive again. At the time it is active, RAS stimulates a protein kinase known commonly as mitogen-activated protein kinase kinase (MAPKKK). In this case, BRAF is MAPKKK, which promotes the phosphorylation of MAPKK (MEK), the second protein kinase in the cascade. MAPKKs have a dual affinity for the residues of tyrosine and serine/threonine amino acids, a property that is essential for cascade activation of the third and final enzyme, MAPK (ERK/MAPK includes double phosphorylation of a tyrosine residue and a nearly adjacent threonine residue to become activated [107].

2.10.2.2 P13K/AKT Signaling Pathway

One of the pathways regulating cell viability, proliferation, and apoptosis is phosphoinositide 3-kinase (PI3 K) signaling. In ASD and Schizophrenia, synaptic

dysfunction is a typical hallmark. In synaptic proteins such as Shank3, Homer, or PSD95 scaffolds, several ASD-related mutations have been identified. ASD is associated with mutations in the neuroligin synaptic adherence molecules (NLGS), which control the balance between inhibitory and excitatory neurotransmission in mammalian brains. Less than 1% of hereditary ASD is responsible for by NLGS mutations. Nonetheless, NLGS receptor mutations such as NRXN1 (Neurexin-1-alpha gene) and CNTNAP2 (Contactin-associated protein-like 2) were identified in ASD [110].

The behavior of PI3 K is also known as a critical neuronal role regulator. Growing evidence indicates the dysregulated activity of PI3 K and downstream signaling as a major contributor to mental illnesses and possible therapeutic targets. Therefore, in mediating the ASD phenotype, PTEN may be an important regulator of this pathway. There is a prevalence rate of around 8% in a clinical cohort of pediatric patients with ASD with mutations in the PTEN gene. There is a higher prevalence rate for developmental delay and/or mental retardation with mutations in PTEN [111,112]. Related variants of the EIF4E gene have been linked with human behaviors associated with ASD, indicating the role of this mechanism in the development of the nervous system [11].

2.10.3 Pro Inflammatory and Cytokine Pathways

In inflammatory diseases of infectious or non-infectious origin, proinflammatory cytokines play a central role. In reaction to injury and other inflammatory events, pathogen-associated molecular patterns (PAMPs) in infection and damage-associated molecular patterns (DAMPs) cause a cytokine cascade initially composed of proinflammatory cytokines. By activation of inflammatory responses, the cytokines help to contain and overcome the inflammatory center. A cytokine group is formed in response to inflammatory stimuli. In the presence of infection or injury, the function of these proinflammatory cytokines is to communicate with the surrounding tissue. The principal proinflammatory cytokines includes, tumor necrosis factor (TNF)-α, the interleukins (ILs) and interferon- γ (IFN-γ). TNF and IL-1 are

believed to play a vital role in forming the local response by cell activation and activating a cytokine cascade as the key mediators of the inflammatory response. Proinflammatory cytokines, such as fever and acute-phase reaction, can invade the systemic circulation and cause activation of immune cells and major changes in host physiology [113].

2.10.3.1 TGF-beta / BMP signaling Pathway

In nervous system production, BMPs are essential and in ASD, their signaling is dysregulated. In activating downstream Smads proteins, BMPs are used and also interact with other signaling pathways such as MAPK, mTOR, Notch, Hedgehog, and Wntt. Distal-less homeobox (DLX) genes are found to be dysregulated in ASD encoding homeodomain transcription factors, resulting in the alteration of BMP signaling. In craniofacial patterning and survival of forebrain inhibitory neurons, DLX genes are involved in. One of the attributes was observed to be overexpressed in a BMP-binding endothelial controller cell line. upregulation, DLX5 [114]. The two subgroups of the superfamily TGF-beta are TGF activin and the bone morphogenetic protein (BMP). The primary subdivision of the TGF superfamily is BMP and are important to the growth of the nervous system. Higher blood serotonin levels (5-hydrotryptamine or 5 HT) were associated with ASD. NLGN4 drosophila neuroligin 4 LOF results in decreased formation of neuromuscular junction (NMJ) with fewer synaptic buttons due to the decrease in type I BMP thickvein receptor, suggesting essential signaling functions for BMP in brains that are natural and autistic. The depletion of the FMR1 protein (FMRP) increases the type II receptor of the bone morphogenetic protein (BMPR2). It has been reported that Ube3a inhibition of BMP signaling plays a role in the activation of synapse growth and endocytosis. ASD has been linked with the DLX genes encoding homeodomain transcription factors. In a cell line that overexpresses Dlx5, The BMP-binding endothelial regulator (Bmper) was found to be upregulated, suggesting that dysregulated DLX function can lead to altered BMP signaling in ASD patients [89]. No association has been reported between TGF-β1 polymorphisms and autism [11]. One of the most omnipresent regulators of

embryonic change, biochemical, and cellular types is the evolving growth figure β (TGF-β) superfamily of cytokines. Three non-synonymous DLX2 mutations ('Ser7', 'GluLys' and 'Ala-Thr') and two in DLX5 ('Ser-Pro' and 'Ser-Arg') have also been found in autistic individuals through DLX gene review of autistic patients and non-autistic siblings [115]. Lower levels of TGF-beta are associated with less adaptive and deteriorating behavioral symptoms in ASD patients. While increase in level of IL-6, IL-8, IL1 beta are associated with impaired stereotypical behaviour [116].

2.10.3.2 JAK/STAT Signaling Pathway

Cytokines are signalling peptides formed by immune and stromal cells that, including the CNS and peripheral nervous system, are involved in the immune response to tissue homeostasis. Some cytokines have also been labelled neuromodulators, in addition to acting as immune regulators. In addition to functioning as immune regulators, certain cytokines have also been labelled neuromodulators. As the nervous system is capable of modulating the immune response, the CNS and the immune system have a finely tuned connection. A study has already found that ASD is associated with transcription factor signaling dysfunction in regulatory cells in Th1, Th2, Th17, and T [11].

Furthermore, studies documented that imbalances between anti & pro-inflammatory conditions are correlated with the production of ASD [117]. A previous study found that in ASD brains, IL-1β and IFN-γ were significantly elevated. The levels of IL-1β, IL-6, and IL-12 in peripheral blood mononuclear cells of ASD patients were shown to be elevated in plasma [118,11].

In the development, maintenance, and survival of glia and neurons in the CNSS, the JAK-STAT pathway was included [117,119]. Some of these molecules have been treated as putative biomarkers, such as IL-6, STAT5, and JAK1, since in ASD patients, numerous JAK / STAT signalling partners and cascade inducing cytokines are changed [11]. JAK-STAT signalling has been shown to improve brain-derived neurotrophic factor expression and in the development and survival

of glia and neurons in the CNS, and is involved. A research recently showed that JAK-STAT communication has greatly increased in children with ASD compared to controls [120].

2.10.3.3 NF-kB signaling Pathway

NF-kB may be a pro-inflammatory pathway that is normally regulated by transmembrane receptors such as TNFR, TLR and other cytokine receptors by different ligands (of microbial origin and endogenous origin). In mammals, five members of the NF-3B family have been identified, including p50, RelA (p65), c-Rel, p52, and RelB, which, in response to separate stimuli, form different combinations of homo- and heterodimers with different DNA binding specificities and transactivation potential. In addition to changes in the immune homeostasis of ASD patients in the brain, variations in NF-kb have been reported in peripheral immune cells [11]. Nuclear factor kappa-B is a nuclear protein presentation in B precursor lymphocyte cells [121].

NF-kB is a critical monitor of healthy capacities, and all of these observations can be explained by improvements in its functions required improvement in the authoritative behaviour of NF-kB DNA of children with severe introvertness in fringe blood tests [122]. In autistic subjects, the amount of IKK kinase that phosphorylates the inhibitory subunit IB is significantly increased in the cerebellum. Besides, previous researches, including ours, have shown that the Bcl2 anti-apoptotic protein decreases, while the p53 proapoptotic protein in the autistic brain increases [123].

2.11 Pathways Crosstalks and Therapeutic Targets

The current study highlights some putative therapeutic targets along these pathways, and further research efforts could grow into novel therapeutic approaches for

some conditions of ASD. Among the different major signaling cascades, Crosstalk often refers to their putative therapeutic consequences. Based on this collective evidence, we conclude that the neurodevelopment and neuroimmune control of homeostatic patterns can be formed by a timely and accurate modulation of these prominent pathways and, hopefully, rescue some ASD phenotypes [11]. Moreover, pathways can affect each other's P-value which is termed as cross-talks. These cross talks occur due to interactions of biomolecules, or gene overlapping among the pathways [28]. The cell responds to its environment through signaling pathways, it consists of many regulatory proteins as well as interactions among cells receptors, intermediate protein complex, transcription factors and target genes. When the external stimulus initiates the receptor some conformational changes occur to intermediate protein which activates the target genes by regulation of transcriptional factors. This stimulation of one pathway may affect the target gene of other pathway, this interlinked connections of pathways is given the name "pathway cross talks". It imparts great effect it developmental processes.

Atypical regulation of pathway crosstalk has been associated to neurodegeneration [36]. Pathway cross talk describes communication between the pathways because biomolecules involved perform more than one part in particular pathway so it may be involved in more than one biological function [37]. ASD genes may be up regulated or down regulated of interlinked Wnt, TGF beta, SHH, and RA signalling pathways [89]. Also the cross talks among Ras, MAPK, P13K-AKT and mTOR effects neural cell differentiation, proliferation and mitosis [11]. Crosstalk between Wnt and Gli has been studied and are potential therapeutic targets in colon cancer [124].

Gli1 negatively regulates GLI3R which inhibits the activity of beta catenin. Loss of function mutation in UBE3A (an ASD associated gene) influences both Wnt and BMP showing possible cross talk between them. It also contributes in RA signalling it mediates neural synaptic plasticity in ASD by negative regulation of ALDH1A (An enzyme of RA synthesis) [89]. Nlgn3 an ASD associated gene, it targets Wnt pathway and also regulates BMP pathway. The signalling crosstalk among these morphogenetic pathways is triggered by autistic genes [92,89]. Wnt

also regulates NF-kB negatively by overexpressing beta catenin that interacts with and inhibits the activity of NF-kB in human breast cancer. This inhibitory effect is also found in non-cancerous cells including fibroblasts, epithelial cells and hepatocytes [125]. MAPKs serve as main synaptic plasticity regulators and PI3K/AKT/mTOR is essential for mental health. Via synaptic plasticity, P13K and Wnt also demonstrate cross chat. AKT, p38 or ERK1/2 phosphorylation may affect activation of the NF-kB pathway in Alzheimer's disease [11]. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is negative regulator of P13K/ AKT, its inactivation germ line mutation develop neurological disorders including epilepsy, autism etc [126]. Hence, the crosstalk among several pathways in neural cells of ASD should be studied further for designing better treatment strategies. Only few genes involved in ASD among nine high confidence genes, have been studied which shows involvement in impaired signalling pathways [89]. For targeting novel therapeutic targets one must know about the molecular level strategies which could be investigated through the pathway analysis involved in neurodevelopmental, immunoregulatory system, and metabolism which might be changed in ASD. Their cross-talks are also important for finding and predicting actual potential targets or biomarkers which by further investigations could be the novel approach of diagnosis or treatment [11].

Chapter 3

Methodology

This study is designed to analyse these pathways. In order to establish a cross talk among major pathways involved in ASD, methodology summarized in 3.1 was exploited.

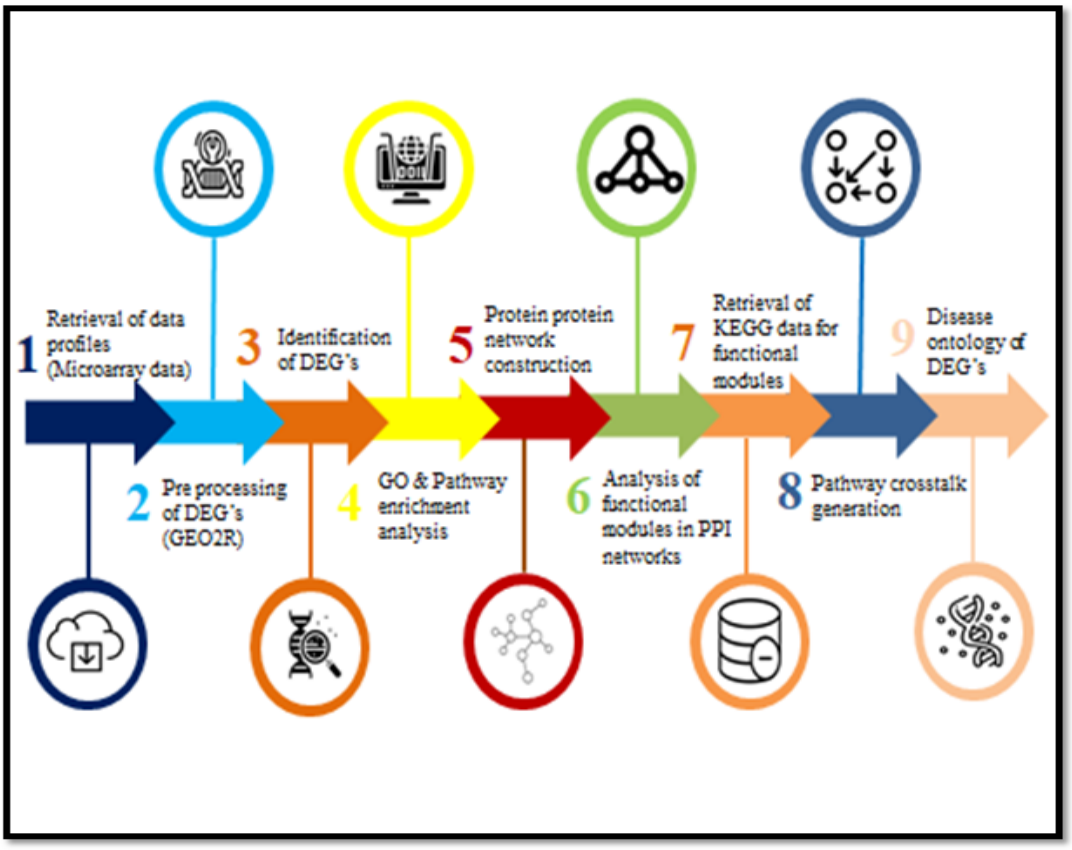

FIGURE 3.1: Methodology used for identification of pathways cross talks in ASD (Flow Chart of Methodology).

Autism spectrum disorder is multifactorial disease occurred due to the alteration in developmental, inflammatory and metabolic pathways. These pathways also show cross talks among them, which would be novel therapeutic targets in future.

The microarray data retrieved which categorizes differentially expressed genes involved in ASD, after identification of these DEGs, gene ontology and pathway enrichment were performed.

Protein protein network construction would help to identify the networks of DEGs. A cross talk would be generated to identify the majorly involved genes among all of the pathways.

3.1 Prioritizing of Key Pathways Reported in Literature to be Involved in Autism Spectrum Disorder

The first objective of the study is to prioritize the key pathways which are involved in ASD from the literature. For this purpose an extensive review were given to the literature.

However few pathways were present, and the selection of pathways were done according to the crosstalks among the pathways involved is ASD. These pathways were identified as developmental pathways, metabolism pathways and pro inflammatory and cytokines pathways.

3.2 Identification of Key Genes Involved in Autism Using Pathway Crosstalks

The second objective of this study is to identify those genes which are involved in the pathways of ASD, for this purpose following steps are performed which are

given below.

3.2.1 Retrieval of Data Profiles

For retrieving the microarray data, National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gds) was used. The volume and range of data maintained by NCBI has grown tremendously and can be grouped into six categories: literature, health, genomes, genes, proteins and chemicals [127].

NCBI provides data on genes in excess of 10 million genes from nearly 10,000 species. In addition to curation by in-house employees, these data are accumulated and retained through many international partnerships. The GEO DataSets database searched can be performed using many different attributes including text, id, keywords, organism, DataSet type and authors [128].

3.2.2 Pre Processing of DEGs

Differentially expressed genes (DEGs) were pre processed by using an automated tool of NCBI, GEO2R. If a discrepancy or shift found in reading counts or expression levels between two experimental conditions is statistically important, a gene is considered to be differentially expressed. For gene expression analysis, several statistical methods are present.

To approximate the model of differential gene expression, these statistical distributions are used. The selection of such genes is based on a mixture of the threshold for change of expression and some cutoff score, typically created by statistical modelling [129]. The Gene Expression Omnibus (GEO) database is an international public repository that stores and freely distributes gene expression and other functional genomics data sets of high-throughput genes.

With many rapidly evolving techniques, it has evolved and now accepts high-throughput data. For tens of thousands of studies, this database provides access to data as well as anticipates different web-based tools and techniques that allow

users to locate data relevant to their particular interests, as well as to visualize and analyze data.

3.2.3 Identification of Differentially Expressed Genes

For the identification of DEGs, GEO2R was used. GEO submissions significantly exceed the rate created for datasets, GEO2R has been developed to provide immediate, web-based, and user-driven analysis for this purpose. It is an interactive instrument that allows approximately 90 percent of the GEO Sequence to be analyzed. It uses a web-based program with the Benjamini-Hochberg false-discovery rate method for multiple-test correction as its default method, using the Bioconductor, GEOQuery and limma packages in R.

The input as disease name in input bar of NCBI GEO2R, clicking the text "Analyze with GEO2R" Using the "Value Distribution" tab to verify the distribution of the sample values. The groups are developed here by clicking on the groups identified as control or disease. Using default parameters and clicking top 250 on the GEO2R page, the analysis output is now completed. The DGEs are displayed in a new window ordered by P-values [130]. In this study the DEGs were identified their default values and some cutoff criteria Benjamini and hochberg method for adjustment of P-values of genes which minimizes the false discovery rate (FDR). FDR < 0.05 and Log2F >1 genes were selected [131].

3.2.4 Gene Ontology and Pathways Enrichment Analysis

To identify the enriched functionally associated genes groups, Gene Ontology (GO) performed using the tool Go Term Mapper (https://go.princeton.edu/cgi-bin/GOTermMapper). Gene Ontology (GO) offers a structure and collection of principles to explain the roles of all organism's gene products. For supporting the computational representation of biological systems it is specifically designed to ensure the system biology needs. It is an interaction between a particular gene product and a GO idea, which together make a notion important to the gene's

function. A molecular function (MF) in the GO is a mechanism that can be carried out by direct physical interactions with other molecular entities by the action of a single macromolecular machine. Function implies an action or operation carried out by a gene product. These activities involve biochemical activity and play a role in a larger system/process as a part.

Although the biological process (BP) reflects a particular target to be accomplished by the organism genetically programmed. For example, the biological process of cell division results in the development of two daughter cells from a single parent cell. The resulting state of each cell defines its BP. Every BP is achieved by a complex collection of molecular processes, often in a highly regulated manner and in a specific temporal series, carried out by specific gene products [132].

In this study the GO term mapper input windows contains uploading genes list tab, choosing ontologies, of MF, BP and cellular components, an ontology option (generic slim, GOA slim), organism selection and results in form of plain text or HTML. Here GO of MF and BP genes were collected for homo sapiens genes, in HTML table format.

3.2.4.1 Pathway Enrichment Analysis

Pathway enrichment analysis helps to provide the insight into genes lists and identifying biological pathways that are enriched in involved genes [133]. PEA were performed by using Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) tool which is a web based annotation tool and grouping of annotation based on functional annotation tool (clustering, charts and tables), gene functional classification tool and gene id conversion tools [134]. DAVID Gene Functional Classification Tool within a few seconds, can manage up to 3,000 genes efficiently at a time. In addition, it also enables one gene to be present in more than one functional community, which closely resembles the essence of biology in which a gene can play several roles in various processes [135]. Using DAVID gene functional classification tools genes clusters were retrieved, in which the pathways having these genes were noted.

3.2.4.2 Malacards Database

Once the pathway with enriched genes had been identified, verifications of these pathways with already existing pathways involved in ASD had been performed using a database named malacards (https://www.malacards.org/).

It is a human disease database, With more than 26,000 diseases affecting all areas of the body, including blood, bone, immune, muscle, and reproductive diseases, the anatomical disease section covers 18 major categories.

In the global disease portion, there are 6 groups: cancer, fetal, hereditary, infectious, metabolic, and rare diseases, and about 18,000 individual diseases. Disease names have been collected from 10 primary disease databases as secondary sources, with another 11 databases.

Overall, the site consists of more than 19,000 disease entries from around 70 major databases and websites, referring to more than 13,000 genes [136].

Pathways related to autism according to KEGG, GO terms (cellular components, molecular function and biological process) had been compare with the pathways retrieved from DAVID. Those pathways which were matched, there genes were saved in tabular form.

3.2.4.3 GenCLiP 2.0

For the DEGs network analysis GenCLip had been used. It is a web based text mining human gene function and network analysis server, which identifies biological functions and molecular networks in a given gene list [137,131]. A default cutoff criteria had been chosen to perform its GO clustering i.e p value $< 1e-4$ *and hit* $>$ 3. Among three modules in GenCLip [131] two of them have been used, Gene cluster with literature profiles, and literature mining gene networks.

Clusters of genes were identified with literature survey and genes, which graphically represent a heatmap. On the other hand in gene network analysis, a network is generated with their respected genes.

3.2.4.4 KEGG Mapper

Kegg mapper is a database used for KEGG mapping, three databases integrated are pathways, (brite, module, network and diseases) which contains the experimental data from published literature and represented in terms of KEGG [138]. The tools were previously made available separately for separate databases, but have been merged into three general mapping tools in the new version. Each of them enables mapping at a time against multiple databases. A type of gene set enrichment analysis, the pathway database is the most commonly used as a reference knowledge base for biological interpretation of user datasets through KEGG pathway mapping [138]. For searching pathways, default parameters were used entering DEGs of ASD, and pathways were selected for homo sapiens and are executed for results. DEGs of DAVID were used as an input for KEGG mapper to cross check the results.

3.2.5 Protein Protein Network Construction

Protein InteraCtion KnowLedgeBasE (Pickle 2.0) is a protein protein interaction integrated meta database it is used for the direct identification of PPIs network in human. PICKLE's special features are the use of the revised human complete proteome (RHCP) UniProtKB/Swiss-Prot as the reference protein collection over which PPI datasets are incorporated [139]. Hence for finding PPIs of DEGs, home page of Pickle gives a window to search the PPIs, second option contains two categories of mouse and human. Moreover DEGs from DAVID had been used, with selection of human in search bar.

3.2.6 Analysis of Functional Modules in Protein Protein Interactions

Network Analyst is a comprehensive web based tool which is designed and used for meta analysis of gene expression data by visualization of PPIs with statistical

evidence [140]. It calculates the values based on centrality. It is an essential index because it shows the nodes in an entire network which occupy a critical position. Betweenness centrality is to quantify the role of 'mediation' in a network by one node undertaking.

If one node is located in the hub through which other nodes, such as contact, link, transport or transaction, have to go through, then this node should be essential and very likely to have a high centrality of betweenness [141].

We have selected PPI of two modules, including generic PPI and microbiome PPI in which network visualization of PPI and their tabular data are fetched. Those genes which betweenness centrality occurs above 1000 for main network were selected, and for subnetworks above 0 genes were selected. Hence final 729 DEGs were retrieved from network analyst.

3.2.7 Retrieval of KEGG Data for Functional Modules

EnrichNet is a tool used for network based enrichment analysis for assessment of functional association of DEGs. It manifests novel graphical based statistical values, which explore the molecular networks connecting two genes set with new visualization of sub networks structure [142].

DEGs of ASD were given as an input in enrichnet tool, we retrieved four different annotated databases results, and compare three databases results with KEGG pathways. Those pathways were selected which were present in all four databases and show cross talks with same DEGs.

3.3 Elucidation of Therapeutic Targets Based on Pathway Crosstalks

The third objective of the study is to elucidate the therapeutic targets based on pathways crosstalks. For this purpose following steps are performed:

Table 3.1: Description of Tools Used in identification of Pathway Crosstalk in Autism Spectrum Disorder.

	Tools Used in Pathway Crosstalk			
Sr.no	**Databases**	**URL**	**Description**	**References**
1	XTalkDB	http://www.xtalkdb.org/home	It is used to mediate cross talks among the pathways, provides easy to use interface for scientists.	[36]
2	Reactome	https://pathme.scai.fraunhofer.de/	It retrieves pathway knowledge from three databases into unified schema to explore pathway cross talks.	[143]
3	BinoX	http://sonnhammer.org/BinoX	It is intended for accurate study of the high throughput of crosstalk gene lists. It reduces the time needed for computation.	[144]
4	CrossTalkZ	https://www.mybiosoftware.com/crosstalkz-statistical-tool-assess-crosstalk-enrichment-node-groupings-network.html	CrossTalkZ is software used to evaluate the importance of crosstalk between pathways. It demonstrates connectivity within/between gene classes.	[145]

	Tools Used In Network Analysis			
1	KEGG	https://www.genome.jp/kegg/	Kyoto encyclopedia of genes and genomes is a database contains three other databases for pathway analysis, gene functions and ligand for conforming enzymatic function.	[146]
2	Reactome	https://reactome.org/	It is a curated database for biological pathway analysis. It generates pathways across 20 species and provide a platform for huge data mining, modelling and analysis.	[147]
3	NCI Pathway	http://pid.nci.nih.gov/index.shtml	It is a collection of curated and peer reviewed pathways (imported form reactome and biocarta) involves regulatory events in key pathways.	[148]

	Tools Used in Protein Protein Interaction Network			
1	PICKLE	http://www.pickle.gr/	Protein InteraCtion Knowledgebase, it is meta database for direct PPI networks in humans. It approaches primary PPI datasets through genetic informations	[139]
2	BioGrid	https://thebiogrid.org/	The biological general repository for interaction datasets is a database of curated protein and genetic interaction data consisting of major model organism from yeast to human.	[149]
3	HPRD	http://www.hprd.org/	Human protein reference database is experimentally derived database containing information about human proteomics i.e. PPI, post translational modifications and tissues expressions.	[150]
4	STRING	https://string-db.org/	STRING is a database of known and predicted protein- protein interactions. The associations involved in this are both physical and functional interactions between proteins.	[151]

	Tools Used in Enrichment analysis			
1	FunRich	http://www.funrich.org/	It is a software tool used for funcational enrichment and interactions network analysis of genes and proteins. It's a user friendly, an open access enrichment tool.	[152]
2	Enrichr	https://maayanlab.cloud/Enrichr/	It is freely available tool, used for enrichment analysis of comprehensive curated genes which interprets biological knowledge.	[153]
3	DAVID	https://david.ncifcrf.gov/	It is a database for Annotation, Visualization and Integrated Discovery. It has different sets of tools through which functional annotation is done to know about integrated pathways behind the genes.	[134]

3.3.1 Pathway Cross Talk Generation

Gephi is an open source network visualization, analysis and exploration tool used for pathway cross talk. The pathways which showed the cross talks were analyze and visualize with the gephi. It uses statistical value and used for visualization and manipulation of large graphs.

3.3.2 Disease Ontology of DEGs

Target mine was used to identify the link between ASD and other diseases. It is an integrated data warehouse. Target mine is able to identify known disease-associated genes with high precision and coverage [131]. The tool has displaying options for genes, proteins, domains, structures, interactions, pathways and compounds. A list of genes was uploaded to target mine. Total of 86 genes from the pathway crosstalks are given to target mine as an input genes, and start analyse. The tool output 57 identifiers, which are added to the tool, as a result disease enrichment window shows the results. The parameters used were benjamini hochberg as default, max P-value was change to 1 to achieve the maximum disease ontolgy of DEGs. Autism spectrum disorder is multifactorial disease which would an outcome of variations in multiple pathways.

Chapter 4

Results and Discussions

In this chapter, the results of each step of the designed methodology are given in details. The results are given in form of tables and figures, with proper description of tools and databases. Moreover, the results of each objective are discussed under separate headings in order to avoid any confusion. For identification of the pathway cross talks involved in ASD, following analysis had been done for finding the hub genes involved in key pathways which could be used for better therapeutic targets in future.

4.1 Prioritizing of Key Pathways Reported in Literature to be Involved in ASD

In order to achieve the first objective of the study, an extensive literature review had been done to prioritize the key pathways which were involved in ASD. For understanding the multifactorial functions of ASD, signalling pathways had been targeted to know the crosstalks of the pathways. These signalling cascades control broader range of neurological features which includes neuronal development, metabolism, homeostasis, and immune regulation and inflammation. Moreover, the signalling pathways involved in ASD had been highlighted in this section which includes, developmental pathways contains Wnt signalling pathway, Sonic

hedgehog signalling pathway, Retinoic acid signalling pathway). Development involves differentiation and structuring of previously unstructured fields. Changes in structures and functions are known to be developmental changes [80].

The human brain undergoes many developmental changes, which causes complex cognitive abilities, these may includes the language, reasoning and cognitive controls. These abilities are made possible by trajectories of brain development [81].

Another type of pathways selected were metabolic pathways highlighting MAP-K/ERK signaling pathway, P13k/Akt signaling pathway. Metabolic pathways involves chains of reaction where the chemical products become the substrate for the next step of process. All substrates of process are chemically modified in reactions that belong to pathways or metabolic cycles [105].

Finally, proinflammatory and cytokines pathways contains TGF-beta signaling pathways, JAK/STAT signaling pathways, and NF-kb signaling pathways [11].

In inflammatory diseases of infectious or non-infectious origin, the proinflammatory and cytokine pathways play a vital role.

By activating the local and systemic inflammatory responses, the cytokines help to contain and overcome the inflammatory center. A cytokine group is formed in response to inflammatory stimuli.

The function of these proinflammatory cytokines is to convey the existence of infection or damage to the surrounding tissue [113].

4.2 Identification of Key Genes Involved in Autism Using Pathway Crosstalks

In this step the second objective of the study had been achieved in which several steps had been performed, for identification of those genes which are involved in the pathways of autism spectrum disorder. For this purpose following steps were performed which are given below.

4.2.1 Retrieval of Microarray Data

In order to retrieve the microarray data of ASD, National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gds) was used. The quantity and range of data maintained by NCBI has grown tremendously and can be grouped into six categories: literature, health, genomes, chromosomes, proteins and chemical [127].

NCBI provides data on genes in excess of 10 million genes from nearly 10,000 species. The NCBI DataSets database searched can be performed using many different attributes including text, id, keywords, organism, DataSet type and authors [128]. The input had been given in search bar as disease name "Autism". In the database selection bar, GEO datasets were selected. As a result a set of raw data of many sets of genes were displayed.

4.2.2 Pre Processing of DEGs

To check the raw data in proper pre process form, differentially expressed genes (DEGs) were pre processed by using an automated tool of NCBI, GEO2R. An international public repository that archives and freely distributes high-throughput gene expression as well as other functional genomics data sets is the Gene Expression Omnibus (GEO) database. For pre processing of DEGs only four data sets were selected as they show the option for "Analyze with GEO2R". After selection of this pre processing data a new window showed the GEO accession, and platform in which more then one data were generated separately for different platform and same accession numbers.

4.2.3 Identification of DEGs

For the identification of DEGs, GEO2R was used, in which after selecting plat forms, groups were created as given by the name in the table as control vs disease. After defining the groups by names given, selection of respective group from the

column group were made. Default parameters were selected from "options" i.e. Benjamini and hochberg method, FDR <0.05 cutoff and start analyse. As a result, the top DEGs were displayed in tabular form with the columns id, adj P-value, P-value, logg FC, gene symbol and gene title. This data were retrieved and saved in the forms of table. In this study the DEGs were identified their default values and some cutoff criteria Benjamini and hochberg method for adjustment of P-values of genes which minimizes the false discovery rate (FDR). FDR < 0.05 and Log FC > 1 genes were selected [131]. Total of 1005 were identified as DEGs for ASD.

4.2.4 Gene Ontology and Pathways Enrichment Analysis

To identify the enriched functionally associated genes groups, Gene Ontology (GO) performed using the tool Go Term Mapper (https://go.princeton.edu/cgi-bin/GOTermMapper). The GO offers a structure and set of principles to explain the functions of gene products from all species, providing genes with the granular GO [132].

The GO term mapper were used for generic annotation of DEGs, the input windows contains uploading genes list tab, ontologies aspect (MF, BP), an ontology option (generic slim) and organism selection (homo sapiens). The output tabular data were in form of gene id, gene annotations for the GO term, the use of the Go term in the gene list and the frequency of use of the genome. Total of 1111 genes were given as an input, in which 202 were duplicated, and about 70 were not found to be annotated.

4.2.4.1 Pathways Enrichment Analysis

Pathway enrichment analysis helps to provide the insight into genes lists and identifying biological pathways that are enriched in involved genes [133]. PEA were performed by using Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (https://david.ncifcrf.gov/) which is a web based annotation tool and grouping of annotation based on functional annotation tool (clustering,

charts and tables), gene functional classification tool and gene id conversion tools [134]. DAVID Gene Functional Classification Within a few seconds, the tool can manage up to 3,000 genes efficiently at a time. In addition, it also enables one gene to be present in more than one functional community, which closely resembles the essence of biology in which a gene can play several roles in various processes [135]. The DEGs were uploaded in input section of DAVID gene functional classification tool, with the selection of homo sapiens species. Functional annotation clustering were used, as a results annotation clusters, enrichment score, P-values, gene counts and benjamini values were displayed in resultant tab. We can calculate E-value, i.e. the number of times we expect to observe a cluster with this score or greater by chance, in genes. Thus, E-values < 1 become increasingly significant, The lower the E-value, or the closer it is to zero, the more the match is "significant" [154]. Here, gene clusters below 0 enrichment scores were selected. For checking clusters 1000 DEGs were given and 117 total clusters were retrieved, but only those clusters were retrieved which were functional to ASD pathways.

4.2.4.2 Malacards Database

Once the pathway with enriched genes had been identified, verification of these pathways with already existing pathways involved in ASD had been performed using a database named malacards (https://www.malacards.org/) [136]. In a search bar disease name "Autism" was searched, as a results 2126 types with their respective scores of autism were displayed. The disease simple identifies autism with score 11.567 were selected.

Pathways related to autism according to KEGG, GO terms (cellular components, molecular function and biological process) had been compare with the pathways retrieved from DAVID. The pathways were identified in DAVID enriched pathways. Total 12 pathways were compared in both databases and saved as functional 5 clusters. The 68 functional genes from both DAVID and malacards were retrieved and saved given in figure 4.1.

Table 4.1: The top 5 clusters gene ontology of differentially expressed genes.

Category	GO term	Hits	P-values
	Negative regulation of canonical wnt signaling pathway.	MAPK14, NOTCH1, PSME3, PSMA6, UBB PSMB10.	9.3E-3
	Positive regulation of canonical wnt signaling pathway.	SRC, PSME3, PSMA6, PSMB10, UBB.	1.5E-2
Cluster 1 Enrichment score: 2.36	Wnt signaling pathway, planar cell polarity pathway.	PSME3, PSMA6, PSMB10, UBB.	3.6E-2
	MAPK cascade	HRAS, KRAS, IL2RG, LAMTOR2, PSME3, PSMA6, PSMB10, UBB.	4.7E-3
	NIK/NF-kappaB signaling	RELB, PSME3, PSMA6, PSMB10, UBB.	1.8E-3
Cluster 2 Enrichment score: 1.57	Erk1/Erk2 Mapk Signaling pathway.	GNAI2, GNAS, HRAS, KRAS, NOS1.	6.8E-3
	Long-term depression	GNAS, HRAS, SRC.	2.2E-1
Cluster 3 Enrichment score: 1.45	MAPK signaling pathway	HRAS, KRAS, RELB, MAPK14, TP53.	4.0E-1
	PI3K-Akt signaling pathway.	HRAS, KRAS, COL4A1, CCNE1, ITGB3, IL2RG, NOS3, PCK1, SPP1, TLR4, TP53.	1.1E-2

Continued Table 4.1: The top 5 clusters gene ontology of differentially expressed genes.

Category	GO term	Hits	P-values
	Cell-cell adhesion	ERC1, HIST1H3A, HIST1H3D, SPTAN1.	5.6E-1
Cluster 4 Enrichment score: 0.21	Cadherin binding involved in cell-cell adhesion	ERC1, HIST1H3A, HIST1H3D, SPTAN1.	6.2E-1
	Cell-cell adherens junction.	ERC1, HIST1H3A, HIST1H3D, SPTAN1.	6.9E-1
Cluster 5 Enrichment score: 0.89	Voltage gated channel	NOX1, KCNMA1, KCNA2, KCNQ5.	1.2E-1

4.2.4.3 GenCLiP 2.0

In order to reveal the DEGs network analysis and GO annotations from literature, GenCLip which is used for human gene function and network analysis had been used. It is a web based text mining human gene function and network analysis server, which identifies biological functions and molecular netwroks in a given gene list [137,131]. A default cutoff criteria had been chosen to perform its GO clustering i.e p value $<$ 1e-4 and hit $>$ 3. Among three modules in GenCLip [131] two of them have been used, Gene cluster with literature profiles, and literature mining gene networks. In order to get the clusters of DEGs 68 genes (an output of 5 enriched clusters genes, from DAVID) was uploaded to input data box, with the selection of identifier as official gene symbol. As a result, gene information, gene cluster with literature profile with above default cutoff criteria, and literature mining gene network were performed. Among 68 genes,32 was given, with 81 clusters having enrichment scores given in form of table as well as a heatmap of that table, and a network of these genes were generated respectively and shown in figure 4.1.

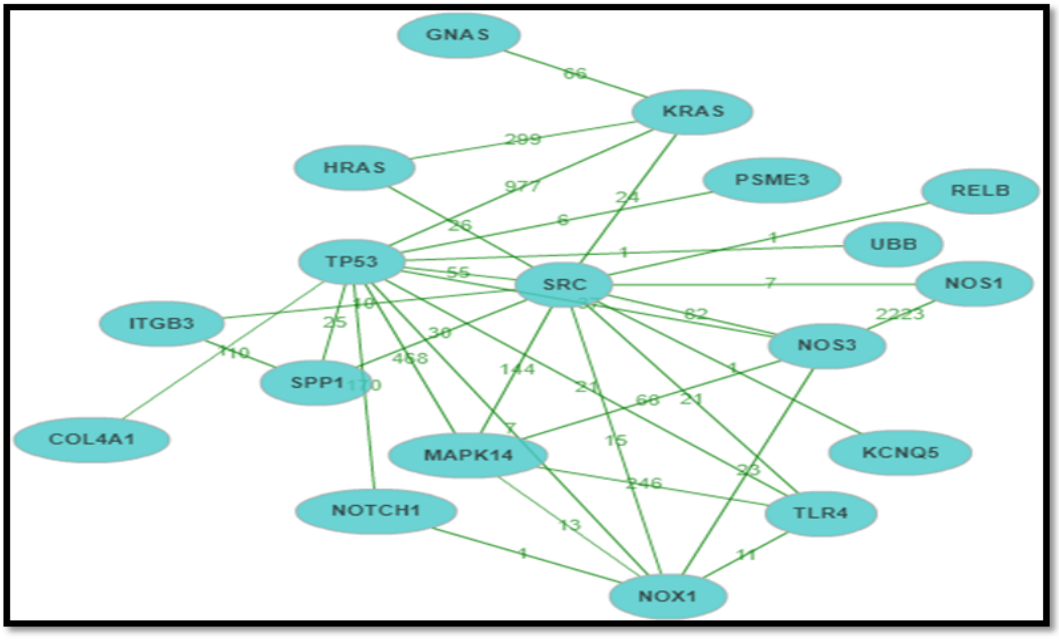

FIGURE 4.1: Total of 18 genes from 32 related pairs of DEGs form a network, the number showing the co cites from literature mining. The number mentioning of abstracts for a particular gene and its pair genes.

4.2.4.4 KEGG Mapper

Kegg mapper is a database used for KEGG mapping, three databases integrated are pathways, (brite, module, network and diseases) which contains the experimental data from published literature and represented in terms of KEGG [138]. KEGG is an interactive collection of 18 databases categorized into four categories: systems, genomics, chemicals, and health information.

KEGG Mapper is a set of KEGG mapping tools for databases of Pathway, Brite, and Module [138]. In order to search pathways, 68 genes were used with default parameters, and pathways for homo sapiens were selected. It reveals three types of pathways, simple pathways with genes, network of pathways and diseases pathways related to DEGs. Against these 68 DEGs, 190 pathways, 64 networks and 86 disease pathways were retrieved.

4.2.5 Protein Protein Network Construction

Once the DEGs pathways enrichment analysis had been done, with different tools their protein protein network construction was done to achieve the results that how proteins interact with each other in differentially expressed genes. In order to get these results, Protein InteraCtion KnowLedgeBasE (Pickle 2.0) were used.

It is a protein protein interaction integrated meta database used for the direct identification of PPIs network in human. Unique PICKLE's features are the use of the revised human complete proteome (RHCP) UniProtKB/Swiss-Prot as the reference protein collection over which PPI datasets are incorporated [139].

Hence for finding PPIs of DEGs, home page of Pickle had an input window, 68 DEGs were inputted to search the PPIs, second option contains two categories of mouse and human PPIs, in this study selection was for homosapiens.

As a result, 3279 interaction was found in tabular form contains, interactor A, interactor B, sources (Biogrid, dip, HPRD, InAct and Mint), cross check confidence and standard confidence.

4.2.6 Analysis of Functional Modules in Protein Protein Interactions

To get the interaction in functional form, Network Analyst was used. It a comprehensive web based tool which is designed and used for meta analysis of gene expression data by visualization of PPIs with statistical evidence [140].

It measure the values on basis of centrality. It is an essential index because it shows the nodes in an entire network which occupy a critical position. It calculates the values based on centrality. Betweenness centrality is to quantify the role of 'mediation' in a network by one node undertaking.

Betweenness centrality is to quantify the role of 'mediation' in a network by one node undertaking. If one node is located in the hub through which other nodes, such as contact, link, transport or transaction, have to go through, then this node should be essential and very likely to have a high centrality of betweenness [141].

We have selected PPI of two modules, including generic PPI and microbiome PPI in which network visualization of PPI and their tabular data were fetched. Hence, 3279 interactions found form Pickle was used, and search had been performed in tab "gene list input".

Once tab open, organism specified for homo sapiens, and set ID type to official gene symbol, and uploaded the genes. Total 4236 of duplicates were replaced and proceed the analysis. Those genes which betweenness centrality occurs above 1000 for main network were selected, and for subnetworks above 0 genes were selected. Hence final 729 DEGs were retrieved from network analyst.

4.2.6.1 Generic Protein Protein Interactions

To get protein protein interations with generic form Rolland interactome with none of parameters and experimentally validated forum was used, in which about 2355 nodes, 5323 edges and 554 seeds in network were given in form of major network and subnetworks. The major network is given in figure 4.2.

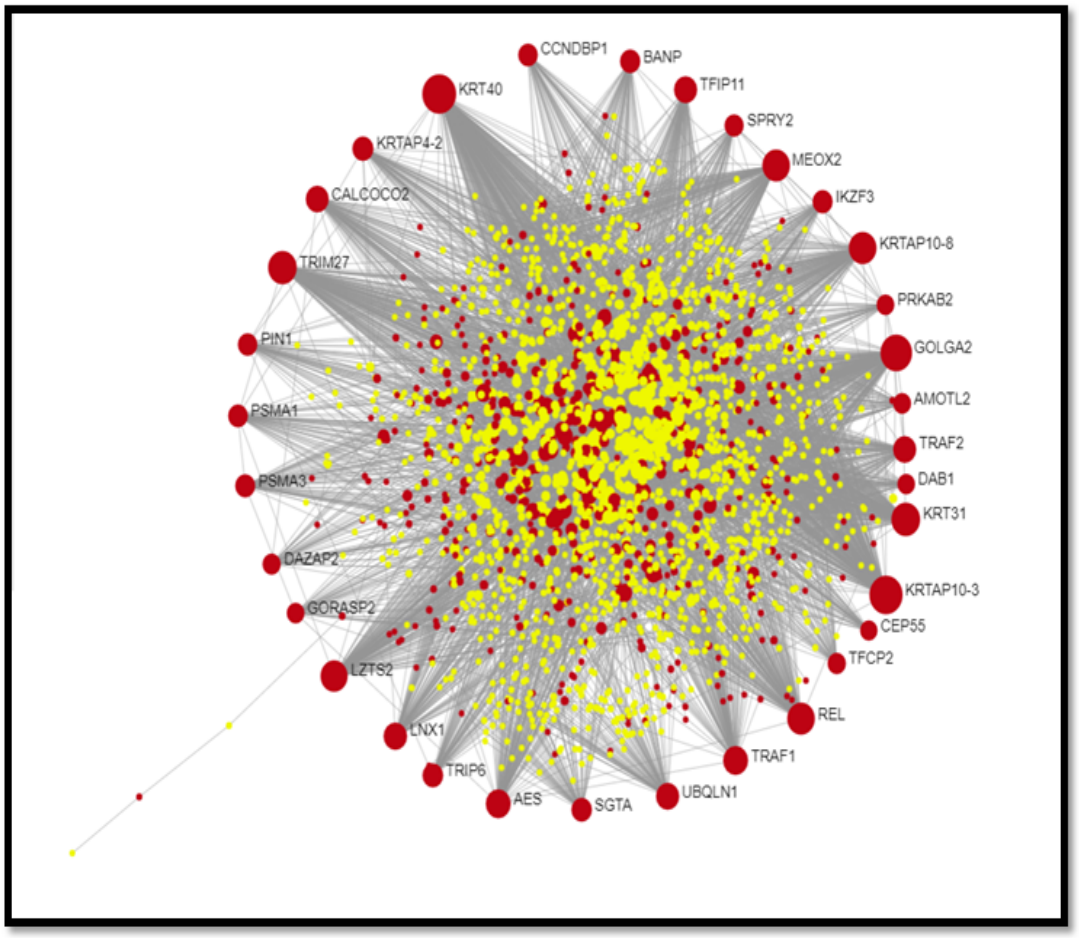

FIGURE 4.2: This pictorial presentation shows a typical view of protein protein interaction network analysis and visualization. There are 2355 nodes, 5323 edges to be built in the network. Nodes represents seed (significant) proteins shown in red color while edges establish relationships among proteins, yellow color showing the proteins interactions in the network.

4.2.6.2 Microbiome Protein Protein Interactions

Similarly for microbiome interactions analysis was done. In this study specie selected was general microbiome, no specific microbiome was selected. As in this study no microbiome selection was done. Parameter selection was microbiolink domain-domain interaction.

In protein-protein interaction data, domain-domain interaction is crucial and important for the better and improved intractome production. As a result 490 nodes, 503 seeds and 1894 edges were given in form of major network given in 4.3.

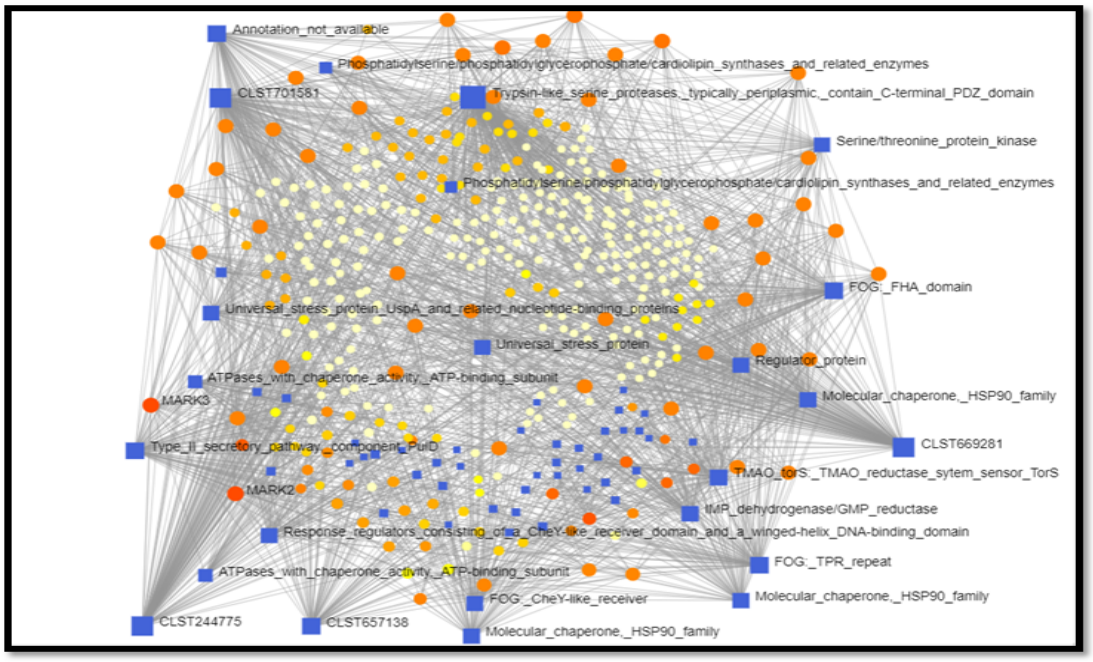

FIGURE 4.3: A typical view of microbiome PPIs shows network analysis and visualization. There are 490 nodes, 503 seeds and 1894 edges. Blue color is highlighting the nodes which are domians of microbiome species interacting with human genes, while the seed nodes in orange and yellow representing the interactions of domians with those of human genes.

4.2.7 Retrieval of KEGG Data for Functional Modules

EnrichNet is a tool used for network based enrichment analysis for assessment of functional association of DEGs. It manifests novel graphical based statistical values, which explore the molecular networks connecting two genes set with new visualization of sub networks structure [142]. In order to get the functional modules of DEGs of ASD, 68 genes were given as an input in enrichnet tool, using its defualt settings of molecular network of string and identifier format as ensembl id and started analysis. Furthermore, annotation databases (KEGG, Biocarta, Reactome and NCI pathways interaction database) were used for analysis of functional module. As a result similarity ranking of gene set vs pathways were displayed with pathways, significance of network distance distribution (XD score), significance of overlap (Fisher test, Q test) and pathways gene set. For retrieval of functional modules, four different annotated databases results, were analysed and compare three databases results with KEGG pathways. Those pathways were selected as

functional which were present in all four databases and showed cross talks among the genes. Hence, 10 pathways were validated from all four databases.

4.2.7.1 KEGG Database

Annotation of pathways with their XD-score had been given in table which shows their network distance distribution, and significance of overlap by fisher test and Q value had been given with the genes set overlaping in a given pathway.

The fisher test, q value and XD score are given in graphical form. The overall genes absolute pearson correlation between XD score and fisher q value is 0.9 and XD score significance threshold is calculated as 0.66.

4.2.7.2 BioCarta Database

The overall genes absolute pearson correlation between XD score and fisher q value is 0.83 and XD score significance threshold is calculated as 2.7 XD score measures the distance of the set the DEGs for pathways.

The importance of the gene set overlap between the pathway and the user-defined gene set is calculated by a Fisher-test in order to equate this score to a classical enrichment analysis score. To assess the importance of the gene set overlap between the pathway and the target gene set, Fisher's exact test p-values are measured.

4.2.7.3 NCI Database

The overall genes absolute pearson correlation between XD score and fisher q value is 0.9 and XD score significance threshold is calculated as 1.19.

4.2.7.4 Reactome Database

The overall genes absolute pearson correlation between XD score and fisher q value is 0.87 and XD score significance threshold is calculated as 1.39.

4.3 Elucidation of Therapeutic Targets Based on Pathway Crosstalks

The third objective of the study is to elucidate the therapeutic targets based on pathways crosstalks. For this purpose following steps were performed:

4.3.1 Pathway Cross Talk Generation

Gephi is an open source network visualization, analysis and exploration tool used for pathway cross talk. The pathways which showed the cross talks were analyse and visualize with the gephi. The directed pathway cross talks had been generated for DEGs of ASD weighted 1. In module, there were 11nodes and 10edges, average density of which were 0.91. The 10 pathways cross talks had been generated, with average degree of 0.909.

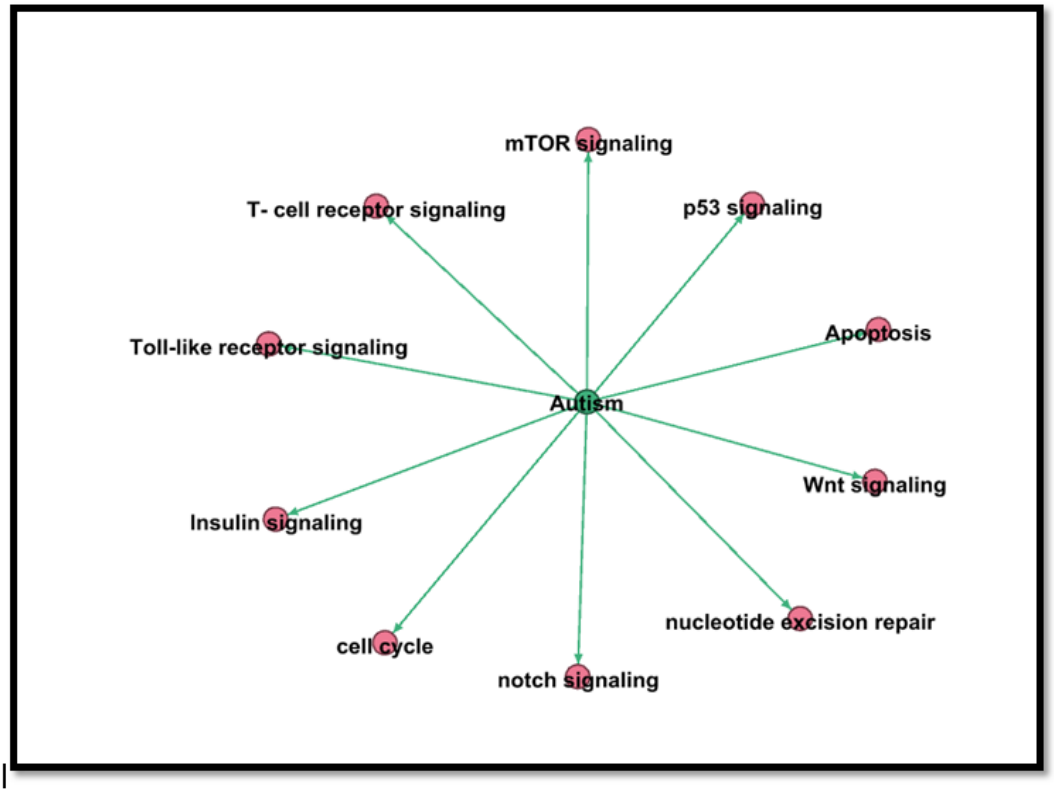

FIGURE 4.4: The green node in the middle is the root node represents host pathway (autism), rose red nodes are pathways interacting with the host pathways.

4.3.2 Disease Ontology of DEGs

For getting the disease ontology of DEGS, Target mine was used to identify the link between ASD and other diseases. It is an integrated data warehouse. Target mine is able to identify known disease-associated genes with high precision and coverage [131]. The tool contains information for genes, proteins, domains, structures, interactions, pathways and compounds. A list of genes was uploaded to target mine. Total of 86 genes from the pathway crosstalks genes uploaded to target mine as an input genes. The tool output 57 identifiers, which were added to the tool, as a result disease enrichment window shows the results of those genes which were enriched in diseases. The parameters used, max P-value was change to 1 to achieve the maximum disease ontolgy of DEGs. The disease ontology of ASD differentially expressed genes involved 749 diseases, which showed the cross domains of DEGs with other diseases.

Chapter 5

Conclusions and Future Prospects

Autism spectrum disorder is a heterogeneous disorder, and manifests in early three to five years of life. In this study bioinformatics, insilico meathods were used to achieve the objectives of study and to explore the mechanism behind the pathway cross talks. For this purpose, three objectives were divided to achieve the results. In order to achieve first objective, i.e. Prioritising of key pathways reported in literature to be involved in autism spectrum disorder, the literature minning for the cross talk of ASD had been done, to priotrize those pathways which are involved in cross talks of ASD majorly. The signaling cascades control broader range of neurological features such as neuronal development, neurotransmission, metabolism, and homeostasis, as well as immune regulation and inflammation. Moreover, the signaling pathways involved in ASD had been highlighted which includes, developmental pathways (Wnt, Sonic hedgehog, Retinoic acid), metabolic pathways (Mapk/Erk, P13k/Akt), proinflammatory and cytokines pathways (TGF-beta, Jak-Stat, NF-kb). To get the second objective of the study, Identification of key genes which are involved in autism using pathway cross talks were done. For this purpose retrieval of microarray data was performed, the total 1000 DEGs of microarray data were retrieved from database NCBI. After DEGs identification, gene ontologies were performed using GO term mapper for generic annotations of DEGs. After the annotations of DEGs pathway enrichment was performed by using DAVID tool and malacard database, from there 5 functional clusters with

their respective enrichment score had been achieved (enrichment score with positive values indicates the genes at the top of the list). In this study DEGs were mostly enriched in GO terms of WNT, MAPk, NF-KB, P13-k/AKT/mTOR, cell adhesion molecules, long term depression and voltage gated channel. It had been discussed that ASD is caused by dysregulation of developmental, metabolic and pro inflammatory pathways.

Furthermore, the pathways already reported for ASD had been retrieved from malacard database and matched, only those genes were retrieved which pathways were matched with enriched pathways. The validated patwhays results in 68 genes, for further analysis. However, for further pathway enrichment GenCLiP was used to identify the networks and pathways based on literature survey. A network of DEGs were presented which gives detail about the genes and literature co cites that how these genes were previously linked with other genes in the network. After pathway enrichment analysis, Protein protein interactions of those DEGs were performed, using Pickle. As a result 3279 interactions were given, which were further analyze for finding functional modules in those interactions. Analysis of functional module based on centrality. For the validation and crosstalk among the pathways, KEGG database with other three databases, BioCarta, NCI and reactome databases were used. These four databases validates 10 pathways, and shows crosstalks among pathways.

In order to achieve third objective of the study, ie elucidation of therapeutic targets based on pathway cross talks. Gephi tool were used to generate the cross talks among the pathways. The directed pathways crosstalks had been generated for ASD weighted 1. In module, there were 11 nodes and 10 edges showed the crosstalks of 10 pathways. These pathways includes, mTOR, p53, apoptosis, wnt, nucleotide excision repair, notch, cell cycle, insulin, toll like receptor and t-cell receptor signaling. Wnt and mTOR pathways were already reported in literature and had some crosstalks with other signaling pathways. In pathway crosstalk network, various novel pathways were identified besides already present pathway crosstalks, these remaining pathways generating crosstalk are identified as new crosstalks of pathways. These novel crosstalks would help us to identify ASD

treatments easily. The 86 DEGs out of 1122 DEGs from NCBI were identified from these novel pathways are considered as target nodes for better and prospective therapeutic targets. Similarly, the DO of these genes also plays crucial role in other diseases, which were not properly linked yet. In future, by exploring the new pathways crosstalk, novel therapeutic targets would be achieved possibily. These targets might be able to cure the core symptoms of ASD. The experimental validation of these targets is also suggested to validate the crosstalks and their role for better treatments of ASD.

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