RESEARCH ARTICLE

A REVIEW ON APPROACHES TO DIAGNOSE THE AUTISM SPECTRUM DISORDER: THE NEED OF THE HOUR.

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Abstract

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Autism spectrum disorder is a complex multigenic neurodevelopmental disorder with a prevalence of 1 in 68 children affecting male more than female (1 in 42 boys and 1 in 189 girls). In present study, focus has been put forth on several aspects of autism diagnosis. To that end association of certain biomarkers such as oxytocin, vasopressin, retinoic acid and 5-hydroxymethylcytosine has been discussed. As autism is also known to be caused by parental influence, prenatal stress, improper uterine environment, drugs taken during pregnancy and role of vertically transmitted diseases has also been given importance. Biological cause correlates autism with various other disorders such as schizophrenia, fragile X syndrome and mitochondrial disorders. Moreover, autism is known to be caused by more than 100 genes. Thus, a thorough emphasis has been given to highlight inter and intra-association of all the relevant diseases and biomarkers to give more insight regarding diagnostic measures of autism.

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Abbreviations:-
AD - Alzheimer's disease
ADHD - Attention deficit hyperactivity disorder
AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASD - Autism spectrum disorder
ALT - Alanine aminotransferase
AMPAR - Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid receptor
AST - Aspartate aminotransferase
AVP - Arginine vasopressin system
BDNF - Brain derived neurotrophic factor
cADPR - Cyclic ADP Ribose
CCL - Chemokine C-C motif ligand
CD - Creatin Deficiency
CD38- Cluster of differentiation 38
CDH9 - Cadherin
CDH10 - Cadherin 10
CHD8 - Chromodomain helicase DNA binding protein 8
CK - Creatine kinase

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DLG - Disks large homolog
DSM - Diagnostic and Statistical Manual of Mental Disorders
DYRK1A - Dual- specificity tyrosine-(Y) - posphorylation regulated kinase 1A
ETC- Electron transport chain
F2-IsoPs - F2t-Isoprostanes
FMR1 - Fragile X mental retardation
FXS - Fragile X syndrome
FXTAS - Fragile X-associated tremor/ataxia syndrome FXTAS
GABAA - Gamma-aminobutyric acid A receptor
GABAB - Gamma-aminobutyric acid B receptor
GABR - Gamma – aminobutyric acid type A receptor unit
GI -Galvanized iron
GluR - Glutamate receptor
GRIK1 - Glutamate Ionotropic Receptor Kainate Type Subunit 1
GRIN2B - Glutamate receptor, ionotropic, N- methyl D- aspartate 2B
GTP - Guanosine Tri-Phosphate
GWAS - Genome wide associated studies
HIV - Human immunodeficiency virus
HFA - High-functioning autism
ICSI - Intracytoplasmic sperm injection
ID - Intellectual disability
IFN- GAMMA- Interferon gamma
 G Immunoglobulin -IgG
IgM - Immunoglobulin M
IL - Interleukin
IL-1RAcP - Interleukin 1 receptor accessory protein
IL1RAPL1 - X-linked interleukin-1 receptor accessory protein-like 1
IVF- In vitro fertilization
KATNAL2 - Katanin p60 subunit A1 like 2
LTD - Long term depression
LCLs - Lymphoblastic cell lines
LTP - Long term potentiation
MAGUK - Membrane-associated guanylate   kinase
MeCP2 - Encoding methyl CpG binding protein 2
MTHFR- Methylenetetrahydrofolatereductase
mTOR - Mechanistic target of rapamycin
NLGN - Neuroligin
NMDA -   N-methyl-D-aspartate
NRXN1 -Neurexin 1
OXT - Oxytocin
OXTR - Human oxytocin receptor
PAX - Paired box
PICK1 - Protein interacting with C-linked kinase
PKU - Phenylketonuria
POGZ - Pogo transposable element with ZNF domain
PSD - Postsynaptic density protein
PTEN - Protein tyrosine phosphatase
RELN - Reelin
Rh- Rhesus factor
RhoA - Member A of the Ras homologue gene family
S100B - S100 calcium-binding protein B
SAH - *S* - adenosylhomocysteine
SAM - *S* - adenosylmethionine
SAP - Synapse-associated protein
SEMA5A - Semaphorin 5A
SHANK3 - SH3 and multiple ankyrin repeat domains 3

SLC6A4 - Human serotonin transporter
SLC25A12 - Solute Carrier Family 25 Member 12
SNC2A - Sodium channel, voltage – gated, type2, alpha subunit
SNP - Single nucleotide polymorphisms
TEMs - Tetraspanin enriched micro-domains
TET - Ten-eleven translocation methylcytosinedioxygenase
TM4SF2 - A member of the transmembrane 4 superfamily, maps to Xp11.4
TSPAN 7- Tetraspanin 7
TAS2R1 - Taste receptor, type 2, member 1
TGF β - Transforming growth factor β
Th1 - Type 1 *T helper*
Th2 - Type 2 *T helper*
Toll/IL-1 - Toll/interleukin-1 receptor
TIR - Homology domain
TPH - Tryptophan Hydroxylase
V1aR - Vasopressin V1a receptors
XLID - X-Linked Intellectual Disability
3CT - 3-Chlortyrosine
3NT - 3-Nitrotyrosine
5hmC - 5-hydroxymethylcytosine
5mC - 5-methylcytosine
8-OHdG - 8-Oxo-2'-deoxyguanosine

INTRODUCTION:-

Autism is a complex multi-gene disorder having overlapping features with many other disorders [1]. The diagnosis of such a complex disorder at an early stage remains elusive even after decades of research. To brief up the entire scenario, focus has been given on two main aspects; one being the cause or risk factors for ASD and the other is the diagnostic criterion. Diagnosis of any disease can be made easier by identifying specific biomarkers, therefore a detailed explanation of various biomarkers and their role in ASD have been discussed. Various factors associated with the incidence of ASD which have been classified into three domains: chemical, environmental and biological influences. A summation of these two topics explains the overall complexity of the disorder and its diagnosis.

Diagnosis of any disorder at an early stage may lead to cure or up to a restricted level. Diagnosis of autism is of a greater challenge as it is known to be influenced by gene, environment and various enzymes and chemicals. The identification of specific biomarkers will help in identifying the disorder at its early stages. Biomarkers are biological characteristics observed in individual's blood or tissues, which are indications of diseased or normal features. Different types of biomarkers are considered for the diagnostic purpose such as genetic, epigenetic, metabolic, oxidative stress, mitochondrial dysfunction, methylation, immune and maternal antibodies etc. Present study is intended to give a thorough overview of present scenario in autism diagnosis and to highlight the all possible aspects associated with autism suitable for futuristic precisely targeted biomarker research for autism diagnosis.

Diagnosis of Autism:-

The search for variations in the genetic makeup of an individual always has an edge over any other diagnostic method. Genetic biomarkers reveal any such change in the affected individual. Taking into account the higher concordance rate of ASD in monozygotic twins (92%) than in dizygotic twins (10%), literature supports a hereditary component in the susceptibility to ASDs [2], which implies gene has its role in ASD. More than 500 different genetic loci are known to play a role in ASD risk [3]. Genome wide associated studies (GWAS) were performed to identify de novo variations associated with ASD. Deletions at the Neurexin 1 (NRXN1) locus, duplications at 7q11.23, duplications at 15q11-13, and deletions and duplications at 16p11.2 were also found to be associated [3]. Earlier studies found rare, functional mutations in genes encoding for NRXN1, SHANK3 (SH3 and multiple ankyrin repeat domains 3), and SHANK2, all of which are proteins that affect the functioning of synapses and have been linked to other known genetic disorders [4]. Synapses are structures in brain that helps in transmission of signal between nerve cells. It has been found that inappropriate numbers and/or morphologies of synapses may lead to a too weakly or too strongly connected network. Such abnormal synaptic connectivity is frequently observed in mental retardation (MR), a condition present in 75% of individuals with ASD [5]. In addition, whole exome

Sequencing verified by several reports have found genetic mutations associated with autism including SNC2A, CHD8, DYRK1A, POGZ, GRIN2B, and KATNAL2 [6]. Common genetic variants on 5p14.1 that are associated with susceptibility to ASDs were also identified by a few other scientists. Among the common polymorphisms found, the methylenetetrahydrofolatereductase (MTHFR) polymorphism is one of the most widely studied genetic correlations with autism [7]. Many other GWAS were performed and a few other genes were identified which include: cadherin (CDH9), cadherin 10 (CDH10), semaphorin 5A (SEMA5A), and taste receptor, type 2, member 1 (TAS2R1), and are found on chromosome 5p14 [8]. Four other large, novel deletions on 2q22.1, 3p26.3, 4q12, and 14q23 that include new genes and regions linked to ASDs were also reported by a few other scientists. The presence of one or many such variant may cause ASD.

Even though concordant rates were observed in monozygotic twins, the severity of the disorder however varied which lead to the role of non-genetic epigenetic factors in ASD. Epigenetics is the study of heritable changes in gene activity that are not caused by changes in the DNA sequence; it also can be described as the study of stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable. Epigenetic changes in ASD occur through methylation, histone modification [9], chromatin remodelling and transcriptional feedback loops [10]. Various studies have shown that DNA methylation differences can occur in many loci including AFF2, AUTS2, GABRB3, NLGN3, NRXN1, SLC6A4, UBE3A [9], the oxytocin receptor [11], MeCP2 (a cause for most cases of Rett syndrome) in the frontal cortex [12], and changed chromatin structure in prefrontal cortex neurons at hundreds of loci [13]. The severity of the autistic phenotype is related to DNA methylation at specific sites across the genome [10]. Environmental and physiological influences are important factors accounting for inter-individual DNA methylation differences, and these influences differ across the genome [14]. These epigenetic biomarkers explain the hereditary component of the disorder. There are no autism-defining, metabolic biomarkers, but examining the biomarkers of pathways associated with ASD points out to potentially treatable metabolic abnormalities and provides a baseline that can be tracked over time. Each child may have different metabolic pathologies related to SNPs, nutrient deficiencies, and toxic exposures. Examples of metabolic disorders that can lead to an autistic-like presentation include phenylketonuria (PKU) [15], disorders of purine metabolism [16], biotinidase deficiency [17], cerebral folate deficiency [18], creatine deficiency [19], and excess propionic acid (which is produced by Clostridium) [20, 21].

Oxidative stress can be detected by studying antioxidant status, antioxidant enzymes, lipid peroxidation, and protein/DNA oxidation, all of which have been found to be elevated in children with autism. Reduced levels of glutathione, glutathione peroxidase, methionine, and cysteine along with increased levels of oxidized glutathione are statistically different in ASD [22]. The level of antioxidants excreted in urine was found to be significantly lower than normal in autistic children. These findings were correlated with the severity of the ASD among individuals [23]. A list of biomarkers and their role have been discussed in brief: Plasma F2t-Isoprostanes (F2-IsoPs) are known to be the most sensitive indicator of redox dysfunction [24]. They are found in higher level in patients with ASD along with gastrointestinal dysfunction. Urine 8-OHdG is biomarker for oxidative damage to DNA. Decreased levels of major antioxidant serum proteins transferrin (iron-binding protein) and ceruloplasmin (copper binding protein) have also been observed in patients with ASD. Plasma 3-chlortyrosine (3CT) and 3-Nitrotyrosine (3NT) levels were reportedly increased with age for those with ASD and mitochondrial dysfunction but not for those with ASD without mitochondrial dysfunction.

Mitochondrial dysfunction is marked by impaired energy production. Some children with ASD have been reported to have a spectrum of mitochondrial dysfunction of differing severity [25]. There is no reliable biomarker to identify all cases of mitochondrial dysfunction [26]. Mitochondrial dysfunction markers include lactate, pyruvate and lactate-to-pyruvate ratio, carnitine (free and total), quantitative plasma amino acids, ubiquinone, ammonia, CD, AST, ALT, and creatine kinase (CK) [25, 19]. Many studies of ASD reported elevations in lactate and pyruvate, or a decrease in carnitine, while others report abnormal alanine in ASD patients [25] or elevations in aspartate aminotransferase and serum CK [27].

The methylation pathway provides methyl groups for many functions, including the methylation of genes, which can result in the epigenetic changes of turning genes on and off. Impaired methylation may reflect the effects of toxic exposure on sulphur metabolism. Oxidative stress initiated by environmental factors in genetically vulnerable individuals can lead to impaired methylation and neurological deficits, both of which may contribute to the manifestation of autism [28]. A marker of methylation dysfunction is decreased SAM/SAH ratio in patients with ASD [29].

Immune biomarkers also play a major role in knowing the onset and severity of ASD. Cytokines are the immune biomarkers and increased level of plasma cytokine is known to exhibit regressive onset and severity of autistic and behavioural symptoms [30]. Altered pro-inflammatory cytokines, complement proteins, chemokines, adhesion molecules, and growth factors are also correlated with ASD. More specifically, altered TGF-beta, CCL2, and CCL5, IgM and IgG classes of immunoglobulin circulating levels are linked with a worsening of the behavioral scores [31]. An imbalance in Th1/Th2 is found as well, which may play a role in the pathogenesis of the autism [32]. Further research has shown that increase in S100B, a calcium binding protein produced primarily by astrocytes, is a biomarker that reflects neurological/brain damage in ASD and its severity [33].

It has been proposed that autoimmune autistic disorder is a major subset of autism [34], and autoimmunity may play a role in the pathogenesis of language and social developmental abnormalities in a subset of children with these disorders [35]. Many auto-antibodies are known to be found in the nervous system of children with ASD [36, 37]. These can be measured as biomarkers in this subset of ASD patients. The anti ganglioside M1 antibodies [38], antineuronal antibodies [39], and serum anti-nuclear antibodies [39, 40] are associated with the severity of autism. Other auto-antibodies that are known to play a pathological role in autism include, anti neuron-axon filament protein (anti-NAFP) and glial fibrillary acidic protein (anti-GFAP) [41], antibodies to brain endothelial cells and nuclei [35], antibodies against myelin basic protein [42,43], and anti-myelin associated glycoprotein, an index for autoimmunity in the brain [44]. High levels of BDNF antibodies were found in ASD patients [45], and low BDNF levels may be involved in the pathophysiology of ASD [46].

There is always an understanding that when the mother is affected by bacteria or virus, the maternal antibodies cross the underdeveloped blood brain barrier of the fetus [47] leading to impaired fetal neurodevelopment and long-term neurodegeneration, neurobehavioral, and cognitive difficulties [48]. Such diseases are called vertically transmitted diseases.

Autism is a neuro-developmental polygenic disorder [1, 49] that affects people with an array of other disorders as well [1, 2, 50]. The actual cause of the disorder is abstruse. In the first half of the article, a focus has been given on identifying or categorizing the disorder with the help of biological markers. The second half will deal with the cause of such a disorder. For a better understanding, the causes have been classified in three main categories: 1) chemical causes 2) environmental causes and 3) other biological causes. Each of which has the influence of genes.
Knowing the influence of certain enzymes and chemicals in ASD is of absolute importance.

1. *Chemical causes of Autism:-*
1.1 GABA and mGluR5: Various reasons have been attributed to the cause of autism and one of which is the decrease in number of synapses with increasing age in autistic children [5]. Glutamate (an excitatory neurotransmitter) and GABA (gamma amino butyric acid), an inhibitory neurotransmitter are known to play a key role in development of synapses and neuroplasticity. Abnormalities in GABA-A receptor ion channels, GABA-B transmitter systems and G-protein coupled metabotropic glutamate receptor (mGLuR) have a link with ASD and its syndromic form. Studies have revealed the role of many signalling proteins that either directly or indirectly modulates mGLuRsignalling (such asneurexin, PTEN, mGluR8, neuroligin 3 and 4). This also causes mGLuRdependant long term depression (LTD). GABA protein is a negative modulator of mGLuR pathway and stimulation of GABA-B has been responsible for decrease in synaptic release of glutamine [51]. However, genetic studies have a different approach towards this.

Genetic Understandings: Neurological and psychiatric disorders are often due to aberrant synapse formation, function, plasticity and malformed dendritic spine. Intellectual disorders are often genetically influenced and a lot of which are X-linked (e.g. alpha thalassemia X-linked intellectual disability syndrome). Two different mutations in the synaptic scaffold proteins have been reported; SHANK family and the MAGUK (Membrane-associated guanylate kinase) family of proteins. SHANK3 is located in chromosome 22 and codes for large postsynaptic scaffold protein SHANK3. It causes Phelan-McDermid syndrome or 22q13.3 deletion syndrome. SHANK genes cause alterations in synaptic morphology, signalling and behaviour characteristics.

Among the MAGUK family of proteins, postsynaptic density protein 95 (PSD-95) is coded by DLG4 (disks large homolog 4) gene in humans. DLG-3 codes for the synapse-associated protein 102 (SAP102) and is linked with ID. It causes premature STOP codons before PDZ domain. The truncated proteins bind to NMDA receptors and other proteins that regulate NMDA pathways.

Mutations in X-chromosome include genes such as IL1RAPL1, Oligophrenin-1andTM4SF2 gene. The IL1RAPL1 gene (interleukin-1 receptor accessory protein-like 1 gene) has many mutations in patients with ID to ASD (non-syndromic). The IL1RAPL1 proteins belong to a new Toll/IL-1 receptor family and shares 52% homology with IL-1 receptor accessory protein (IL-1RAcP). It is a postsynaptic protein and specifically binds to post synaptic density protein-95 (PSD-95). Thus alterations in these proteins due to mutations can cause ID and ASD. The Oligophrenin-1 gene mutations or deletions in the synaptic Rho GTPase-activating protein also cause several modifications. Oligophrenin-1 has relation with XLID(X-linked ID), indicating alterations in function of member A of the Rashomologue gene family (RhoA), implicated in ID. It interacts with the postsynaptic adaptor protein homer. Alterations in oligophrenin-1 expression result in various deficits of synaptic function and plasticity. TheTM4SF2 gene codes for tetraspanin 7 (TSPAN 7) proteins, is an evolutionary conserved protein associated with numerous proteins in the so called tetraspanin enriched micro-domains (TEMs) of the plasma membrane. Mutation in this gene causes premature stop codon TGA (glu218-to-ter) and a 2bp deletion (564 delGT) resulting in premature stop codon in position 192 which is directly associated with ID. TSPAN7 is a synaptic protein that regulates the association of PICK1 (protein interacting with C-linked kinase) with AMPARs and controls AMPAR trafficking [52].

1.2 5-hydroxymethylcytosine: 5-hmC is considered as sixth base of DNA and plays many roles such as chromatin modification and down regulation of gene expression. It varies in concentration depending on the location of the cell and is present in all the cells [53- 55]. Engrailed-2 gene expression plays an important role in histone lysine methylation and acts as a transcriptional repressor during the start of post natal period until perinatal period where it gets down regulated. Over expression of EN-2 was found in autistic cerebellum (from post mortem sample). This gene also plays a role in development of serotonin and other neurotransmitters. It is crucial for the Purkinje cell maturation. In individuals affected with autism, the down regulation of this gene is hindered. 5-mC (hypo methylation) is converted into 5-hydroxymethylcytosine in the presence of ten-eleven-translocase during TET mediated 5-mC demethylation. MeCP2 binds to 5-mC and 5-hmC in a similar fashion. The conversion of 5-mC to 5-hmC also occurs due to oxidative stress during postnatal period in cerebellum. The concentration of 5-hmC is associated with the over expression of EN-2. There is a significant increase in the levels of 5-hmC, DNA methyltransferases, TET dioxygenases in autistic individuals compared to control [54].

Concentration of 5-hmC is high in autistic individuals and MeCP2 binding is decreased in autistic individuals along with overexpression of EN-2. There are six PAX5 binding sites in the upstream of EN-2 gene where 5-hmC concentration is high and it activates the enhancer. MeCP2 binds only to oxidized bases. Here the 8-oxo-dG is responsible for oxidative stress in the bases leading to conversion of 5-mC to 5-hmC and reducing the MeCP2 binding. Therefore, it is clear that there is an overall inter-relation between one another compounds discussed above which ultimately leads to the disorder [54].

1.3 Oxytocin, Vasopressin and CD38: Oxytocin (OXT), a nonapeptide secreted by axons and dendrites of the oxytocinergic neurons located in the paraventricular and supraoptic nuclei of the hypothalamus [56-60] regulates social behaviors, increased gaze, inferring the emotions of others, generosity, trust and face recognition [61-66]. Moreover, OXT is critical in regulating positive social interactions for an individual's mental and physical fitness.

CD38 is a type 2 transmembrane protein having varied functions such as control of leukemia malignancy, as a HIV marker in blood and is known to play a role in social behaviour [67]. It possesses ADP-ribosylcyclase activity that produces cyclic ADP-ribose (cADPR) from beta-NAD+ (vitamin-B3). cADPR is a potential intracellular messenger that helps in cell migration and a co-factor for the movement of Ca^{2+} through Ca^{2+} permeable membranes [68].

CD38 SNPs (R140W found in Asian population and rs3796863 found in American HFA and in Israel) are proposed to be a possible reason behind oxytocin disruption leading to autism [67, 68]. Polymorphism in V1Ar gene in human AVP system is also known to be associated with ASD [69]. Posterior pituitary peptides, including AVP, act centrally as regulators of learning and memory thus any polymorphism or change in their expression will lead to psychiatric problems [70].

1.4 Retinoic acid: Autism prevalence in males boosts the fact that elevated testosterone level in the foetus can be a prompting factor for ASD. Retinoic acid related orphan receptor alpha (RORA) is functionally relevant to ASD. It is dysregulated by environmental factors (gene-environment interactions) and is also hormone sensitive [71]. Thus it suggests that the endocrine disrupting molecules mimic the sex hormone and interfere with normal expression posing a greater risk of autism. RORA is a relevant candidate gene for ASD because its expression is reduced in

ASD especially in subtype associated with severe language impairment based on gene expression profiling of lymphoblastic cell lines (LCLs) [72]. Moreover, reduced expression of RORA increases methylation in RORA promoter in LCL individuals with ASD and can be reversed by exposure of the cell to a methylation inhibitor. It has been found that RORA protein is reduced in post-mortem cerebellum and frontal cortex of individuals with ASD, also dihydrotestosterone is known to decrease RORA expression while estradiol increases it [73]. RORA regulates transcription of aromatase which converts testosterone to estrogen. Level of aromatase is also less in post-mortem cerebellum and frontal cortex of autistic individuals [74]. RORA has sexual dimorphism. It helps in cerebellar development, Purkinje cell differentiation, and regulation of multiple genes at glutamatergic synapse. In addition, RORA is associated with protection of neurons against oxidative stress and inflammation and in regulation of circadian rhythm [75-79].

These are the enzymes and chemicals reported for the cause of autistic traits and hence the disorder. Beside this, environmental factors might contribute greatly to ASD either individually or in conjunction with genetic and/or chemical factors.

2. Environmental causes for Autism:-
Considering the environmental aspects, parents and their lifestyle mainly parental age, maternal stress, migration during pregnancy, maternal exposure to toxicants, uterine environment, vertically transmitted diseases during gestation etc. are the few stated imperative factors that might lead to the risk for autism [80-90].

2.1 Parental influence: Couples having children in late thirties might develop greater risk for autism for childrens. Prenatal stress of mothers, migration during pregnancy, exposure to pollution also largely contributes for the disorder. Gestational diabetes, gestational bleeding, multiple birth, being first born compared to being 3rd or after are the other condition when the offspring may be affected [80]. Maternal antibodies (including cytokines) can cross the blood brain barrier of the foetus and can cause neurological damages; this is due to infections to which the antibodies respond and along with infection it also causes damage to the foetus.

Elevated serum IFN-GAMMA, IL-4, IL-5 is common in mothers who give birth to autistic child. Foetal IL-4 exposure in late pregnancy causes abnormalities in hippocampal structure and morphology and decreased learning in adulthood [81].

2.2 Prenatal stress: Prenatal stress of mothers' affects the child's neurological development [82] though the actual genetic influence or correlation between the genes and prenatal stress and its role in ASD is unknown.

2.3 Uterine environment: Uterine environment is one of the proposed reasons for the risk of autism though the mechanism is still unknown. In vitro childbirth techniques IVF and ICSI too have an effect on the child's growth and development. During perinatal period, prematurity, abnormal presentation in general and breech presentation in particular and planned caesarean sectioning too have an influence. In neonatal period, umbilical cord complications, low 5-min Apgar score, being small for gestational age, low birth weight (<1500g), foetal distress or meconium aspiration as well as birth injury or trauma, summer birth, feeding difficulties, neonatal anaemia, ABO or Rh incompatibility and hyper-bilirubinemia are found to be significantly associated with ASD [83]. Perinatal effects such asanaesthesia during pregnancy, assisted vaginal delivery, post-term birth and high birth weight or head circumference are reported to have an effect on foetus [83].

2.4 Drugs taken during pregnancy or before: In utero exposures, two known teratogenic medicines that are thalidomide and valproate have proven to affect the foetus with mental disabilities. Folic acid and vitamin D (calcitriol) consumption have known to prevent spina bifida. The enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) converts folate into a form where it can be used for methylation. Increase in the intake of folate results in mutation in MTHFR, C677T mutation which increases the risk of autism. Yet folic acid helps in proper methylation, the children with MTHFR mutation and doesn't receive folic acid have a risk of developing autism [84]. Deficiency of Vitamin D leads to dysregulation of 36 proteins in the brain [85]. Calcitriol (activated vitamin D) can down regulate harmful cytokines in the brain, partially reverse brain damage and increase cellular level of antioxidant glutathione which removes free radicals and chelates heavy metals including mercury [86-89].

2.5

2.6 Infections during pregnancy: Maternal infections during pregnancy also increase the risk of autism. Maternal antibodies sometimes attack the foetus brain as a result of immune response or autoimmune disorders [90].

ASD has overlapping features with many other disorders thus the demarcation between ASD from the other is a matter of challenge amongst scientists. A mixture of genetic, chemical and environmental causes also leads to these reported disorders.

3. Biological causes for Autism:-

3.1 Schizophrenia: Several studies reported that individuals involved in violence and aggressive behaviour suffers from psychosis [91-94] in most cases and it has also found that their background is associated with factors such as sexual abuse, low income, use of drugs and alcoholism [91, 92, 95-97]. Affected individuals showed poor speech and reasoning skills which might be a neurocognitive deficit [98]. They exhibit reduced brain activity in right hypothalamus and right superior temporal gyrus region. Same misbehaviour patterns have also been observed in autistic children in some cases which might be associated with poor theory of mind [98]. These two disorders often overlap in their identity and common in males. Autism has a prevalence of 4.1:1 and schizophrenia with 1.4:1 for male. However, there are various ways of differentiating these two disorders with the age of onset and language impairment. Autism onset is during early childhood whereas that of schizophrenia is after onset of puberty. In autism there is severe and profound language impairment whereas in schizophrenia there is a wide array of neuropsychological disorders including language. It is hence clear that autism and schizophrenia has non-genetic causes as well [99].

3.2 Fragile X syndrome: FXS is known to be associated with ASD and is a monogenic disorder that is linked to silencing of FMR1 gene [100]. FXS is the most common genetic cause of ASD, accounting for 5% of cases [101,102]. There are 15-60% of reported cases of ASD in males with FXS. ASD in FXS is a selective disorder of core socialization skills that is relatively independent of general and verbal cognitive skills [100]. Among the genetic disorders associated with ASD, the behavioral phenotype has been best characterized in FXS [103]. Illustrating the central role of adaptive socialization in ASD in FXS is the fact that, whereas most males with FXS and ASD have impaired socialization skills, only those with severe autistic symptoms also show prominent social withdrawal [104]. It has also been observed that social avoidance becomes a prominent component of ASD in FXS after age 5 [100]. Studies however have demonstrated that ASD diagnosis and autistic behaviours are relatively stable over time in FXS [105-107]. A unique relationship between ASD and anxiety in FXS has been postulated, in part, because of the high prevalence of anxiety in individuals with the genetic disorder. Around 75% of young males with FXS are known to display excessive shyness and social anxiety and 50% have panic attacks [108], whereas there are reports that females with FXS also have excessive shyness, social anxiety, and avoidance personality [109]. Other studies emphasize that although individuals with FXS are interested in social interaction, they often display anxiety- and withdrawal-like behaviors in response to unfamiliar people and novel situations [110-111]. Although studies describe general features of individuals with FXS who meet DSM-IV criteria for different anxiety disorders, they are not specific regarding social anxiety and its delineation from the diagnosis of ASD. Many mouse models have also been developed to study the association between ASD and FXS. The relationship between FMR1premutation (i.e., intermediate level expansion of the CGG polymorphism), usually a carrier status that is not associated with atypical methylation or gene silencing, and clinical manifestations has been one of the most controversial ones in the FXS literature [100]. At present, it is well accepted that two disorders, fragile X-associated tremor/ataxia syndrome (FXTAS) and primary ovarian insufficiency, are linked to FMR1premutation [112].

3.3 Mitochondrial disorder: The mitochondria have two membranes, inner (contains the ETC) and the outer membrane. The mitochondrial genome codes for 13 of the ETC proteins [113]. The cell nucleus encodes other mitochondrial proteins (more than 1000) which mediates processes such as the regulation of ion homeostasis, stress responses, cell survival and signal transduction [114]. Neurons depend on mitochondrial energy supply as they have limited capacity to obtain energy through glycolysis when oxidative phosphorylation is compromised [115]. Studies reported that increased mitochondrial mass is required for neuronal differentiation and suggested that mitochondria plays active roles in synaptic plasticity [114]. Events in learning and memory processes such as long term potentiation (LTP), has been associated with changes in mitochondria [116, 117]. Mitochondria works as mediations of some of the effects of glutamate and brain derived neurotrophic factor (BDNF) on synaptic plasticity [118]. Studies reported that patients with psychiatric disorders exhibit mitochondrial abnormalities at the structural, molecular and functional levels. Mitochondrial disorders are associated with psychiatric complications, bipolar

disorder, depression, anxiety disorder etc. and are more common in ASD. ASD patients display peripheral markers of mitochondrial energy metabolism dysfunction, such as elevated lactate, pyruvate and alanine levels in blood, urine and/ or cerebrospinal fluid, serum carnitine deficiency and/ or enhanced oxidative stress [113].

Other disorders such as Rett syndrome, Angelman syndrome, tuberous sclerosis, cerebellar ataxia and cerebellar palsy etc., also have overlapping features with ASD [119].

Apart from these three domains synaptic cleft- influence of genetic and environmental factors is also considered as risk factor for ASD.

Individuals with autism have differences in brain connectivity. Different areas of the brain coordinate with each other in different way. Synapses are the physical connections through which brain cells communicate with each other and synapses decrease as children grow (normally). The number of synapses is usually high in teenagers but decreases in adults. However teenagers with autism possess more synapses than is typically. Mutation in the gene for tuberous sclerosis increases the risk for autism in people. Rapamycin decreases the number of synapses and improves social behaviours. It is called sirolimus and is used in patients with organ transplants to prevent rejection [120]. However, it has severe side effects including suppression of immune system, lung inflammation and risk for diabetes thus cannot be recommended to autistic children. A very similar drug, everolimus is being studied in patients with TS, a genetic disorder that causes tumours, cognitive impairment and increased risk for autism. Lack of pruning in autistic individuals is that, the brain cells of these individuals were filled with damaged parts and deficient in signs of a normal breakdown pathway called "autophagy". In mouse, pruning defect related to a protein called mTOR. When mTOR is overactive, brain cells lose much of their self-pruning ability. Rapamycin is known to inhibit mTOR and showed effectiveness even in older mice that had fully developed autism-like behaviour [121]. More than 100 genes have reported in cases with autism. Mostly associated ones are RELN, SLC6A4, GABR, NLGN, OXTR, MET, SLC25A12, GRIK1 or GluR6, Glyoxalase 1, TPH1 and TPH2 [122]. (Table 1)

CONCLUSION:-

In present article, multifarious causes of ASD have been discussed with major emphasis on three major domains along with various biomarkers. The actual cause for ASD might be only genetic or only environmental or more than one reason may remain involved. However, there are other cases wherein these mutations or modifications may not have any implications on the individual. Owing to its complexity, the actual reason and the start point of these risk factors have not yet been discovered. Even for those that have been stated regarding the cause of autism is still not prevalent in all reported cases of ASD. This explains the necessity of further research to be carried out even in already stated causes of ASD and also on further new ways of diagnosis.

Conflict of Interest:-
Authors declare no conflict of interests among them.

Table 1:-Implications of various genes in ASD

Gene	General role	Role in ASD
Reelin (RELN)	Extracellular glycoprotein that help in migration of neural cells and in establishing neural connections. It plays an important role in positioning neuronal cells in the inferior olivery complex, cerebral cortex, cerebellum in the early embryonic stages.	Reduced levels of this protein and its m-RNA concentration were observed in the Autistic individuals. It is located at 7q22 (an autism critical region). A GGC repeat polymorphism immediately after 5' ATG was observed in RELN gene.
Human serotonin transporter (SLC6A4)	It localizes to chromosome 17q11.1-q12 and consists of 15 exons.	A HTTLPR polymorphism was found in promoter region of SLC6A4 gene. It is identified by elevated blood serotonin level due to no activity of serotonin inhibitors as a result of mutation.
Gamma-aminobutyric acid receptor (GABR)	Chief inhibitory neurotransmitter in the brain, acts by binding to GABA receptor.	Located in 15q11-q13 which is complex region in the genome involved in gene expression, genome instability, imprinting and recombination. Duplication of this region was observed in many ASD individuals.
Neuroligin (NLGN)	Family of cell adhesion molecules that are essential for functional neuron synapses formation.	A *de novo* mutation in NLGN4 leads to premature STOP codon which causes premature termination of protein. A C TO T transition was also observed in NLGN3 which changed a highly conserved arginine residue into cysteine residue; this was inherited from the mother.
Human oxytocin receptor (OXTR)	Synthesized in the hypothalamus; it acts as a neuromodulator in the CNS. It also plays a role in social and repetitive behaviors.	Mutation in this gene increases the risk of autism.
MET	Encodes a trans membrane receptor tyrosine kinase of the hepatocyte growth factor/scatter factor (HGF/SF). Primarily it is an oncogene, however plays an important role neurodevelopment.	Impaired MET leads to abnormal interneuron migration and signaling between them.
SLC25A12	Located in the 2q31 region, encoding mitochondrial/glutamate carrier (AGC1) a key protein involved in ATP synthesis and mitochondrial function.	Several SNPs have been reported, yet the findings so far are not conclusive.
Glutamate receptor gene (GRIK1 or GluR6)	Located at chromosome 6q21, as the glutamate is the principle excitatory neurotransmitter in brain	The actual relation unknown but it is known to be associated.
Glyoxalase 1	Cytosolic, ubiquitously expressed zinc metalloenzyme involved in scavenging toxic alpha-oxoaldehydes formed during cellular metabolic reactions.	GLO1, A419 polymorphisms can lead to autism.
TPH1 and TPH2	It encodes rate-limiting enzymes that control serotonin biosynthesis.	Susceptible gene for ASD. TPH1 is expressed peripherally, however TPH2 in found in brain tissue.

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