Autism Spectrum Disorder: Characterization of Risk Factors, Comorbid Conditions and Herbal Medicines

THESIS

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CERTIFICATE

This is to certify that the thesis entitled ***"Autism Spectrum Disorder: Characterization of Risk Factors, Comorbid Conditions and Herbal Medicines"*** was submitted by **M. Madhu Poornima, ID. No. 2010PHXF802H** for the award of Ph. D. degree of the Institute embodies the original work done by her under my supervision.

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Abstract

Autism Spectrum Disorder (ASD) is known as one of the most challenging disabilities for children and their families. The onset and development of ASD shows huge variability in clinical manifestations, communication deficits like the use of non-verbal behaviors, lack of age appropriate friendship development, inability to share enjoyment or interests, and deficits in social or emotional reciprocity. Additionally, children with ASD may display restricted, repetitive and stereotyped patterns of behavior and activities. Also, children with ASD show presence of comorbid conditions like Attention deficit/ hyperactivity, eating disorders, anxiety, depression, aggression, self-injury, abnormal sleep patterns etc. which often lead to behavioral problems. Incidence of Autism Spectrum Disorder (ASD) is increasing across the globe and no data is available from India regarding the risk factors of ASD.

Earlier reports on direct link of *in utero* disturbances with aetiology of ASD necessitate the evaluation of parental, prenatal, perinatal and neonatal factors for the risk of ASD. In this regard, we proposed a questionnaire based epidemiological assessment to understand the prenatal, perinatal and neonatal risk factors of ASD and comorbid conditions in India. Our study involved designing and validation of questionnaire. A questionnaire was designed based on the probable risk factors of ASDs from existing literature and those specific to Indian environment. Validity of the questionnaire was tested with a small convenient sample followed by construct and reliability testing. The data obtained concluded that the questionnaire was a good instrument for psychometric research. Using the designed questionnaire, data was collected on risk factors and was compared with the observations from equal number of controls. A total of 29 factors were evaluated by unadjusted and adjusted analysis in this study.

Among the risk factors analyzed in our study, *parental factors* like advanced maternal age, consanguinity among parents, polycystic ovarian syndrome and maternal hormonal interventions were very significant risk factors for ASD, while advanced parental age and maternal thyroid hormone imbalance did not contribute towards ASD risk. Out of the *prenatal factors* considered, fetal distress and gestational respiratory infections were found to be associated with ASD. Evaluation of *perinatal and neonatal factors* showed labor complications, pre-term birth, neonatal jaundice, delayed birth cry and birth asphyxia to be associated with ASD. These results advocate additional focused investigations on physiological and genetic changes contributed by these risk factor inducing environments.

Apart from typical symptoms, children with ASD also show the presence of comorbid conditions. But due to lack of awareness about the comorbid conditions of ASD, and the existence of several cultural beliefs and myths in India, it becomes important to understand and educate parents and professionals about these conditions. This awareness will help in designing effective intervention programs for these children. In our study population, children with ASD had hyperactivity, sleep disturbances, skin allergies, food allergies and constipation problems as comorbid conditions. Apart from existing independently, we found that more than one comorbid condition coexisted with ASD symptoms in Indian population. Our study aims to create better awareness among physicians and care takers about the comorbid conditions and projects the need for designing therapies, keeping in mind, the economic, care-giving, and psychosocial perspectives.

In India, ayurvedic herbal medicines are administered as part of disease management for many neurodevelopmental disorders like Autism spectrum disorder (ASD) and linked comorbid challenges. The biochemistry of the ASD and behavioral abnormalities as observed in comorbid conditions is already reported to involve neurotransmitters like GABA, Serotonin and Dopamine. With non – availability of published reports about the cellular and

molecular effects of herbal medicines given to children with ASD, our study aimed to evaluate the effect of ayurvedic medicines on neurotransmitter levels using IMR 32 cell lines. Herbal medicines like Brahmi, Brahmi vati, Brahmi ghritam and Saraswata ghritam increased the neurotransmitter levels in differentiated IMR 32 cells. Since decreased levels of neurotransmitters are observed in behavioral abnormalities as well as ASD, these ayurvedic medicines when prescribed to children with ASD might alleviate the abnormal behavioral symptoms by maintaining neurotransmitter homeostasis.

Table of Contents

Abbreviations & Symbols

A	Alpha
5-HT	5-hydroxytryptamine (Serotonin)
Ach	Acetylcholine
ADHD	Attention-Deficit-Hyperactivity Disorder
aOR	Adjusted Odds Ratio
AS/AD	Asperger's syndrome/ Asperger's Disorder
ASD	Autism Spectrum Disorder
BD	Bipolar Disorder
BM	*Bacopa monnieri*
BV	Brahmi vati
CARS	Childhood Autism Rating Scale
CDC	Centers for Disease Control and Prevention
CDD	Childhood Disintegrative Disorder
CI	Confidence Interval
CAM	Complementary & Alternative Medicine
CVI	Content Validity Index
DA	Dopamine
DCD	Developmental Coordination Disorder
DMSO	Di-methyl sulphoxide
DSM	Diagnostic and Statistical Manual of Mental Disorders
E	Epinephrine
FSH	Follicle Stimulating Hormone
GABA	γ – Amino Butyric Acid

GI	Gastro-Intestinal
GnRH	Gonadotropin – Releasing Hormone
hCG	Human Chorionic Gonadotropin
HCOOH	Formic Acid
hMG	Human Menopausal Gonadotropin
HPLC	High Performance Liquid Chromatography
ICD- 10	International Classification of Diseases – 10
ID	Intellectual Disability
IHEC	Institutional Human Ethics Committee
IL – 1	Interleukin – 1
IUGR	Intra Uterine Growth Retardation
ISAA	Indian Scale for Assessment of ASD
LCMS	Liquid Chromatography Mass Spectroscopy
LD	Learning Disability
LH	Luteinizing Hormone
LOD	Limit of Detection
LOQ	Limit of Quantification
M	Melatonin
MS	Mass spectrometry
NCCS	National Centre for Cell culture
NE	Norepinephrine
NFHS	National Family Health Survey
OCD	Obsessive Compulsive Disorder
OR	Odds Ratio

P	Probability
PCOS	Poly Cystic Ovarian Syndrome
PDA	Photo Diode Array
PDD	Pervasive Developmental Disorders
PDD-NOS	Pervasive-Developmental Disorder – Not Otherwise Specified
RTT	Rett's Disorder
SIB	Self-Injurious Behaviour
SHBG	Sex Hormone Binding Globulin
SSRI	Selective Serotonin Re-uptake Inhibitors
TNF	Tumor Necrosis Factor
UFLC- ESI – MS	Ultra-fast liquid chromatography – electrospray ionization mass spectrometry
WHO	World Health Organization

List of Figures

List of Tables

CHAPTER 1

GENERAL INTRODUCTION

1.1. What is ASD?

Autism Spectrum disorder (ASD) is a neuro-developmental disorder characterized by complex difficulties in behavior and development. The word "autism" comes from the combination of the Greek words "auto" meaning "self" and "ism" meaning "the act, state, or theory of". The word 'autism' initially was linked to detachment from reality in individuals with schizophrenia (Bleuler, 1911). It took its modern sense in 1938, when it was referred to as "autistic psychopaths"(Asperger, 1938). Kanner in 1943, elaborated the term as "an isolated self" in which a person shows restrictive social interaction (Kanner, 1943). Hans Asperger's work was later summarized by Lorna Wing (L. Wing, 1981) as Asperger's syndrome, and later translated into English by Uta Frith (Frith, 1991). Wing pointed out that, there were many similarities in the clinical portrayals of the children described by Kanner and Asperger. Based on the findings of a study conducted with Judith Gould, Wing proposed the triad of impairments for ASD which includes impairments in social interaction, communication and repetitive behavior (Lorna Wing, Gould, & Gillberg, 2011). The concept of the autism 'spectrum' was thus born, describing every aspect of impairments which could vary in severity, and could be present in individuals with different levels of intellectual functioning. This triad was widely adopted and is used into diagnostic criteria till date (M Rutter & Schopler, 1987; Michael Rutter & Schopler, 1992).

1.2 Classification of ASD according to Diagnostic and Statistical Manual

ASD is known as one of the most challenging disabilities for children. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM) IV (1994) and IV TR (2004), autism and related disorders were collected under an umbrella of PDD - Pervasive Developmental Disorders (DSM-IV, 2000) (Fig. 1.1). PDD consists of five disorders with

manifestations in childhood ranging from severe form Autism, through a milder form called Asperger's Disorder and to some aspects of a "broader autism spectrum" called Pervasive-Developmental Disorder – Not Otherwise Specified (PDD-NOS)/atypical autism. According to DSM-IV and International Classification of Diseases – 10 (ICD- 10), two rare conditions, Rett's disorder and Childhood Disintegrative Disorder (CDD), are also included in PDD.

But now, according to DSM 5 (APA, 2013) four previously separate disorders are grouped into a single condition called ASD with different levels of symptom severity. Thus, according to DSM 5, ASD now includes autistic disorder (autism), Asperger's disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified.

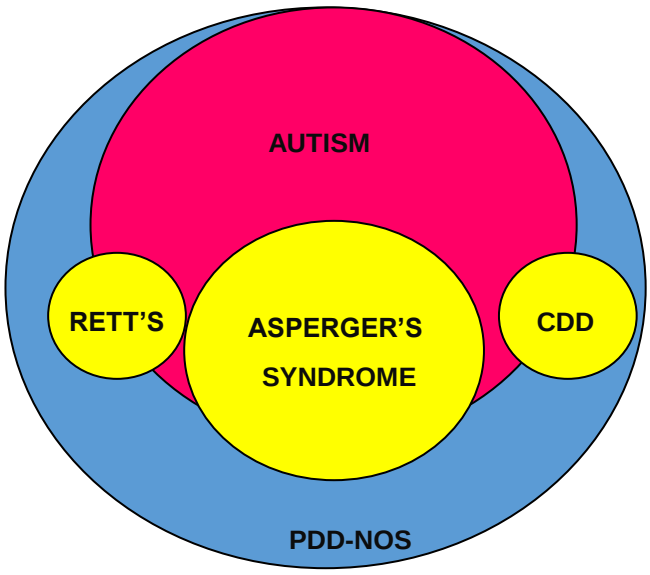


Fig. 1.1 Classification of ASD

Onset, development and phenotypic presentations of ASD show a huge variability with two main types: wherein (i) early manifestation by deviating from the normal development or progression or (ii) sudden regression later, sometimes triggered by an environmental event

(e.g. immune or toxic exposures) after a period of normal development during the first few years. The most typical early symptoms reported for ASD are lack of mimicry and expressiveness (suggesting early abnormalities of motor functioning) (Teitelbaum et al., 1998) and abnormal responses to sensory stimuli (Ornitz et al., 1978; Ornitz, 1988; Gillberg, 1989). The most frequent *clinical manifestations* seen in a child diagnosed with ASD are difficulties in social communication. Additionally, children with ASD may experience restricted, repetitive and stereotyped patterns of behaviour and activities that might not be considered normal (APA, 2013). Moreover, ASD has been characterised as a spectrum of difficulties in these areas that vary in combination and severity, between and within individuals (Charman, 2002).

Autism or Classic autism is a severe form of PDD with impairments in all the three areas: reciprocal social interaction, communication, and restricted, stereotyped, repetitive behaviour. In addition to these specific features, nonspecific problems are also observed, such as sleeping and eating disturbances, phobias, temper tantrums, and self-directed aggression (APA, 2013; DSM-IV, 2000).

Children diagnosed with *Asperger's Disorder (AD)* have difficulty in social interaction, reciprocity and communication. In comparison to autistic disorder, there is no clinically significant general delay in language for diagnosed children with AD. Also, these children do not have clinically significant delays in cognitive development, self- help skills, and in adaptive behaviour except in social interaction, and environmental curiosity (APA, 2013). By age two and three, children use single words and communicative phrases respectively.

A child is diagnosed with *Pervasive Developmental Disorder - Not Otherwise Specified (PDDNOS,* if he/she has a pervasive impairment in the development of reciprocal social interaction. PDD-NOS is a diagnosis of exclusion, which means that the child should

not meet the specified criteria for other developmental disorders such as Schizotypal Personality Disorder, Schizophrenia, another Pervasive Developmental Disorder, , or Avoidant Personality Disorder (APA, 2013).

The prevalence of *Childhood disintegrative disorder (CDD)* is reported to be very low, thus not making any difference in the overall prevalence rates (Fombonne, 2002). In CDD, there is normal development till the first 2 years after birth followed by severe regression and clinically significant loss of previously acquired skills (DSM-IV, 2000). Now, CDD is no longer part of ASD diagnosis according to DSM 5 (APA, 2013).

In *Rett's Disorder* (RTT), significant impairment in expressive and receptive language development with severe psychomotor retardation (DSM-IV, 2000) is observed. These multiple deficits are observed after a period of normal growth following birth. There is a loss of previously acquired hand skills resembling hand wringing. There is also loss of interest in the social environment in the first few years after the onset of the disorder. RTT is reported to be caused due to mutations in the gene MECP2 (Amir et al., 1999) on X-chromosome. It is no more part of ASD as per DSM 5 (APA, 2013).

In our study, we case ascertainment for data collection was done by DSM IV, DSM IV *TR* based ASD diagnosis.

1.3. Aetiology of ASD

Genetics play an important role in ASD, and concordance rates among monozygotic twins and families (Bailey et al., 1995) indicate a strong genetic component. However, multiple genes have been implicated (Buxbaum, 2009) and the extent to which environmental factors are also involved is unclear. This suggest a possible role of both genetic and environmental factors in the etiology of ASD (Bailey et al., 1995; Vincent Guinchat et al., 2012a; Parner, Schendel, & Thorsen, 2008). However, geneticists have not identified a common genetic

mutation that is involved in most cases of ASD. The so far identified common de novo point mutations are reported to be associated with only a small population (Cannell & Grant, 2013). It is also clear that environmental factors, such as infectious diseases and *in utero* teratogen exposure, can cause ASD or there might be an interaction between genetic and environmental factors (Gillberg & Coleman, 2000).

The extreme complexity in the behavioral, developmental and associated medical conditions across ASD indicates existence of multiple unknown causal factors. There is evidence that 54% of the increase in prevalence is a result of known factors, including changes in diagnosis, increased awareness and parental age and the remaining 46% currently unaccounted for (Weintraub, 2011). However, establishing a concrete pathogenesis of ASD has proven to be extremely difficult. The neuropathology of ASD remains unclear and the reported brain abnormalities among children with ASD indicate a probable link with disturbances in the *in utero* period (Gardener, Spiegelman, & Buka, 2011; Minshew & Williams, 2007; Pardo & Eberhart, 2007).

It has been acknowledged that there are a number of comorbidities frequently identified in individuals with autism. These can be of genetic, neurodevelopmental, mental, or behavioural origin or resulting from environmental exposure. Among the most common comorbidities are the learning/intellectual disabilities (LD/ID), epilepsy, tics, Attention-Deficit-Hyperactivity Disorder (ADHD), developmental coordination disorder (DCD), Obsessive Compulsive Disorder (OCD), Bipolar Disorder (BD), anxiety disorders, depressive disorders, Anorexia nervosa, sleep disorders, disruptive behaviour, self-injurious behaviour (SIB), impulse control problems, conduct disorders, feeding problems, constipation, catatonia and mutism, foetal alcohol syndrome etc.. (Coleman & Gillberg, 2012). In addition, there may be co-occurring physical health problems accompanying ASD. Recent research has demonstrated that there are several medical conditions that are significantly more prevalent in

individuals with autism compared to typically developing populations, for example eczema, allergies, asthma, ear and respiratory infections, gastrointestinal problems, severe headaches, migraines and epilepsy (Kohane *et al*., 2012).

1.4. Prevalence of ASD

Globally, since the 1990's, a number of systematic population surveys and routine monitoring systems in various countries have indicated a rise in prevalence from 0.7% to 1% (Chakrabarti, 2001; Elsabbagh et al., 2012; Fombonne, 2002) (Table 1.1).

Generally, it is estimated that ASDs affect up to 60-65 individuals per 10,000 in a population (Elsabbagh et al., 2012). The details about prevalence rates across the world (Elsabbagh et al., 2012) is described in Table 1.1. According to Centers for Disease Control and Prevention (CDC) - recent study from 14 communities, 147 per 10,000 children in the United States have been identified as having an Autism Spectrum Disorder (ASD) (Baio, 2014), indicating a rise in the past two decades. But this may not be the true picture as the prevalence estimates from Asian countries like China (9.8 per 10, 000) Japan (10 per 10, 000) and South Korea (94 per 10, 000) vary widely across time and country (Kim et al., 2011; Sun & Allison, 2010).

Table 1.1 Global prevalence rates of ASD

Country	Prevalence (per 10, 000)
UK	98
Scotland	44.2
Denmark	7.2
Germany	1.9
Israel	48
China	9.8
Japan	10
South Korea	94
Indonesia	11.7
Sri Lanka	107
UAE	29
Iran	6.26

Based on the studies in Asian countries (Kim et al., 2011; Sun & Allison, 2010), nearly 1.7 – 2 million individuals are estimated to be affected (Karande, 2006a; Vibha Krishnamurthy, 2008) with ASD in India.

1.5. Risk Factors of ASD

There has been a huge focus on pre- and perinatal events as risk factors for ASD in various studies across the globe. Studies based on concordance rates among monozygotic twins and families suggest a possible role of both genetic and environmental factors in the etiology of ASD (Guinchat et al., 2012; Parner et al., 2008; Bailey et al., 1995). For example, pregnancy-

induced central nervous system insults may result in relevant epigenetic changes. Secondly, the neuropathology of ASD remains unclear and the reported brain abnormalities among children with ASD indicate a probable link with disturbances in the *in utero* period (Gardner et al., 2011; Minshew & William., 2007; Pardo & Eberhart., 2007). Thirdly, the proportion of children with a major gene defect is limited to a small proportion of ASD cases. Thus, a multifactorial approach towards ASD risk may serve as a more appropriate perspective in the study of the aetiology of ASD. Finally, identification of environmental factors for autism during pregnancy carries clinical implications in terms of primary prevention. Hence, it is imperative to focus of prenatal, perinatal events as risk factors for ASD.

Various risk factors were studied to elucidate their risk towards ASD aetiology, wherein disruptions and disorders of pregnancy, significantly higher incidence of bleeding during pregnancy (Brimacombe et al. 2007; Juul-Dam et al. 2001), breech presentation and low Apgar scores (Larsson et al. 2005), threatened abortion (Hultman et al. 2002), cesarean delivery (Glasson et al. 2004; Hultman et al. 2002) and gestational age at birth\35 weeks (Larsson et al. 2005) or\37 weeks (Brimacombe et al. 2007; Williams et al. 2008b) were predominant. Prenatal exposures to thalidomide, rubella, and daily smoking in early pregnancy were also reported to be associated with an increased risk of ASD (Hultman et al. 2002; Rodier et al. 1996; Stromland et al. 1994). Higher risk for autism has been noted with the presence of one or more unfavorable obstetric events (Bolton et al. 1997; Bryson et al. 1988; Deykin and MacMahon 1980; Finegan and Quarrington 1979). Both advanced paternal age (Durkin et al. 2008; Reichenberg et al. 2006; Shelton et al. 2010) and advanced maternal age (Bilder et al. 2009; Durkin et al. 2008; Shelton et al. 2010; Williams et al. 2008b) also have been reported to be associated with increased risk of ASD. However, the literature is not always consistent with regards to which specific prenatal and perinatal risk factors are associated with ASD.

Out of the various risk factors studied globally, this thesis focuses on relevant ones pertinent to Indian population.

1. *Parental factors*: Consanguineous marriages, advanced maternal and paternal age at the time of child birth, maternal hormonal interventions before conception, conditions like thyroid malfunction and polycystic ovarian syndrome were analysed.

2. *Prenatal characteristics*: Conditions during pregnancy like gestational diabetes, high blood pressure, gestational infections like Urinary tract, Gastrointestinal and Respiratory tract infections, fetal distress inducing conditions like amniotic fluid loss, bleeding during gestation and other suboptimal intrauterine conditions were studied.

3. *Perinatal characteristics*. Labor characteristics like Induced or prolonged labor, Pre-mature membrane rupture; Breech presentation, Nuchal cord, and delivery types including forceps or vacuum suction mediated delivery were analyzed in the study.

4. *Neonatal characteristics*. These included the birth weight and gestational term, birth asphyxia, delayed birth cry, neonatal jaundice, eczema and seizures immediately after birth.

In addition to the above risk factors, the present thesis also focuses on the comorbid conditions of ASD. Apart from typical symptoms, children with ASD also show presence of comorbid conditions like Attention deficit/ hyperactivity, eating disorders, anxiety, depression, aggression, self-injury, abnormal sleep patterns etc. (Matson & Nebel-Schwalm, 2007) which often lead to behavioral problems (Leyfer et al., 2006). We have focused only on factors which are reported to have link with behavioral issues. It is indeed necessary to educate parents about the probable coexistence of these conditions as these may aggravate the ASD challenge. Awareness about these conditions would play a very vital role in choosing early and better intervention for the child.

1.6. ASD in Indian Scenario

- Autism is becoming a growing challenge in developing countries like India as well, and the earlier notion of it being uncommon is no longer justified.
- It poses a much greater and serious challenge in countries like India, because of the severity of the impact on the affected individuals and their families, along with the economic burden that it imposes coupled with lack on scientific know how about the disorder (Daley, 2004).
- Due to lack of awareness about the condition, often, misdiagnosis or inclusion of ASD under the general category of mental retardation and/or speech and language disorders is commonly noticed (Singhi & Malhi, 2001).
- India is country with various regional, religious, social and economic populations. Evaluation of ASD in India is important due to various reasons like a) higher rate of inbreeding in population, b) economic burden imparting stressful life which in turn is contributing to imbalances in hormonal levels in women, c) unavailability of epidemiological data about ASD and d) lack of awareness about the disorder.

Consanguineous marriages are prevalent among Indian families. Various studies consistently implicate a risk of medical complications associated with consanguineous marriages like increased risk of adverse perinatal outcomes including

- ***Stillbirths*** (Hussain, Bittles, & Sullivan, 2001; Khoury & Massad, 2000; Stoltenberg, 1999),
- ***Low birth weight*** (LBW)(Benson, 2005; Hussain et al., 2001; Jordan, 2007; Khalid, Ghina, Fadi, & Fadi, 2006; Khoury & Massad, 2000; Mumtaz et al., 2007; Sezik, Ozkaya, Sezik, Yapar, & Kaya, 2006; Stoltenberg, 1999).
- ***Preterm delivery*** (Al-Eissa & Ba'Aqeel, 1994; Mumtaz et al., 2010),
- ***Apnoea of prematurity*** (Tamim, Khogali, & Beydoun, 2003),

- ***Infant and child mortality*** (Bittles & Black, 2010),
- ***Congenital birth defects and malformations*** (Lutfi Jaber et al., 2005).

Apart from these the risk that an autosomal recessive disorder for a progeny of a consanguineous union is also well characterized (Bittles, 2008). ASD is reported to involve high susceptibility genes and if such genes have autosomal recessive manner of transmission then could be a contributing factor. *But there has been no study done to assess the role of consanguinity and ASD.*

Research reports on epidemiology of ASD from India are not available (Sharan, 2006). According to March of Dimes, fetal distress inducing conditions and complications during labor are documented to be prevalent with significant impact on survival and development of children in India (March of dimes, 2012; NFHS -3., 2007; Singh et al, 2007; Kumar et al, 1996). Mental stress among Indian women due to the race of economic survival in the society, in turn, has reflected in imbalances in hormonal levels, menstrual cycle, infertility treatments, stressful pregnancies (UNICEF, 2006), and probable risk of ASD.

Managing ASD becomes much more difficult when there is association of comorbid conditions like Attention deficit/ hyperactivity, eating disorders, anxiety, depression, aggression, self-injury, and abnormal sleep patterns etc., which are not accounted by ASD diagnosis itself. ASD is a pervasive condition which requires a wide array of services like health & educational (Daley, Singhal, & Krishnamurthy, 2013). Knowledge about these conditions in India is important due to (i) existence of cultural beliefs which sometimes delays ASD diagnosis (ii) the absence of enough intervention services and service providers in India (iii) availability of nominal funding services, concessions or benefits from the Government of India (Aluri & Karanth, 2002; A. Gupta & Singhal, 2005). *There are no reports available in India regarding the comorbid conditions associated with ASD.*

Presence of comorbid condition is the major contributor for parental agony due to behavioural issues. These conditions are sometimes intertwined with each other. Reports suggest that perception of safer usage of ayurvedic medicines are encouraging parents to opt for these alternate medicine for the treatment of comorbid conditions (Hanson et al., 2007). Herbal medicines are administered as part of ayurvedic treatment and no published literature is available regarding the herbal medication given to children with ASD.

Herbal medicine is one style of complementary and alternative medicine. Herbal medicines are standardized herbal preparations consisting of complex mixtures of one or more plants. As a result, there remains a dearth of knowledge concerning the activity of herbal products (Woolf, 2003). This necessitates understanding their role at molecular and cellular levels. The therapeutic efficiency and safety of many herbal plants have been scientifically proved in the past 10yrs (Calixto, 2000). However, there are number of herbal plants of high value among the enormous repertory of indigenous drugs (Calixto, 2000). According to the WHO (X Zhang, 1998), herbal drugs contain as active ingredients plant parts or plant materials in the crude or processed state plus certain excipients, i.e., solvents, diluents or preservatives. In developing countries, a large proportion of the population (>70%) has been reported to still rely on alternate medicine practitioners and their traditional medical knowledge and methods in order to meet health-care needs (Pan et al., 2012; Vaidya & Devasagayam, 2007) which could be due to cultural and/or economic reasons. Because of the healing nature of plants, herbal medicine has been used from past to present to treat illness and diseases, such as the neurological, digestive or respiratory systems (Winston, 2003). Table 1.2 lists out various herbs prescribed to treat neurological disorders (Vaidya, 1997).

Table 1.2 Various herbal medicines used to treat neurological disorders.

Analgesics	**Antidepressants**	**Learning & Memory**	**Anti convulsants**	**Antistress agents**	**Antiparkinson's**	**Anxiolytics**
Corchorus depressus	*Mucuna pruriens*	*Bacopa monniera*	*Withania somnifera*	*Ocimum santum*	*Mucuna pruriens*	*Withania somnifera*
Embeliribes	*Saraca indica*	*Centella asiatica*	*Convolvulus Pluricaulis*	*Eleutherococcus senticosus*	*Kava, evening primose*	*Azadirachata indica*
Gossypium indicum	*Withania somnifera*	*Butea frondosa*	*Erythrina variegata*	*Centella asiatica*	*Ginkgobiloba*	*Nardostachys jatamansi*
Azadirachata indica		*Celastrus paniculates*	*Pongamia pinnata*		*Fava bean*	*Acorus calamus*
Psidium guava		*Eclipta alba*			*Belladonna, Hen bane, Thornapple, Oat Passion flower Jimson weed.*	*Celostrus paniculatus*

Due to the prevailing comorbid conditions in ASD and perceptions of safe usage of alternate medicine i.e., a belief that there are fewer side effects and it being natural by many families, encourage its wider usage for the treatment (Hanson et al., 2007). Other motivating factors for the choice of alternate medicine therapies by the parent may be the promise of treatment of significant comorbid symptoms, such as gastrointestinal difficulties, that are typically not acknowledged by their primary health care provider (Barnes, Powell-Griner, McFann, & Nahin, 2004; Hanson et al., 2007; Levy & Hyman, 2005)

Neurotransmitters like Serotonin (5-HT), Gamma Amino Butyric Acid (GABA), Dopamine (DA), Epinephrine (E) and Norepinephrine (NE), Melatonin (M) and cytokines like Interleukin – 1 (IL1) and Tumor Necrosis Factor (TNF) levels play a very important role in regulation of behavioral issues (Durant, Christmas, & Nutt, 2010; Ernst, Zametkin, Matochik, Pascualvaca, & Cohen, 1997; Hosenbocus & Chahal, 2012; Kindregan, Gallagher, & Gormley, 2015; Murphy, Lerner, Rudnick, & Lesch, 2004; Yanofski, 2010), like sleep regulation (Jouvet, 1972; Krueger, Obál, Fang, Kubota, & Taishi, 2001; Longordo, Kopp, & Lüthi, 2009), hyperactivity - ADHD (Blum et al., 2008; Gold, Blum, Oscar-Berman, & Braverman, 2014) and stress, depression, anxiety (Graeff, Guimarães, De Andrade, & Deakin, 1996). Imbalances in neurotransmitters and cytokines are also implicated in the etiology of ASD (Ashwood et al., 2011b; Oades, Dauvermann, Schimmelmann, Schwarz, & Myint, 2010; Tsai, 1999).

Due to non-availability of scientific data to prove the exact action of herbal medicines prescribed to alleviate behavioral abnormalities (as observed in children with ASD), on living cells, it is necessary to test the impact of herbal medicine on neurotransmitter production at cellular level. *Though age old tradition, limited information is available regarding the action of these medicines on neurotransmitter production.*

1.7. Scope and Objective of the work

ASD is a neurodevelopmental disorder and due to the non-availability of published reports, it is necessary to explore the prenatal, perinatal and neonatal risk factors of ASD from a large ethnic country like India. Since it has both genetic and environmental causal factors, it becomes important to explore the risk factors worldwide. Globalization and women empowerment has led to a change in the lifestyle of Indian women wherein there is integration of education, erratic work routines and household duties, leading to a stressful life. This stress is implicated in obstetric complications leading to problems during prenatal, perinatal and neonatal period. With increase in ASD prevalence and obstetric complications the need to explore the risk factors in Indian population gains importance. Apart from typical ASD symptoms presence of various comorbid conditions is observed among children with ASD. These behavioural comorbid conditions which we have focused on might aggravate ASD challenge if not addressed and thus, poses greater challenges and puts more pressure on the parent or caretaker. Due to the presumed side effects of allopathic medicines, parents are looking for alternate medicines like Ayurveda for the treatment of these behavioral issues. With neurotransmitters playing a significant role in the behavioral issues and ASD, Herbal medicines are reported to ameliorate these behavioral conditions. Due to the availability of very little information regarding the cellular and molecular effects of these herbal medicines on neurotransmitter level, preliminary cell culture based work was carried out in IMR 32 cells.

Objectives

1. Identification, enlisting and statistical evaluation of the risk factors associated with ASD in India through a case- control cohort study.
2. To study the presence of various comorbid conditions in ASD
3. To evaluate the effect of herbal medicines on neurotransmitter levels in IMR 32 cell line.

To achieve these objectives the following work plan was carried out.

1. Development and validation of the questionnaire

   Questionnaire was validated for its content and reliability. Statistical approach was used to evaluate the results using SAS software.
2. Characterization of parental, prenatal, perinatal and neonatal risk factors of ASD

   Regression analysis was performed to calculate the odds ratio and results are represented in statistically significant figures.
3. Characterization of comorbid conditions
4. Evaluation of effect of herbal medicines on neurotransmitter level in IMR 32.

   The LCMS method developed was also validated with calibration curves, limit of detection and quantification, repeatability, accuracy and matrix effect. This method was tested to evaluate the effect of various herbal medicines on neurotransmitter levels in IMR 32.

CHAPTER 2

DESIGNING AND VALIDATION OF QUESTIONNAIRE USED TO ASSESS RISK FACTORS OF AUTISM SPECTRUM DISORDER

2.1. Why is questionnaire an important tool?

Autism Spectrum Disorder (ASD) is a multifactorial neuro developmental disability associated with extremely complex behavioural, developmental and medical issues (Guinchat et al., 2012). Despite significant research in the field, the aetiology of ASD and the multiple causal factors are not well established.

Data across the globe especially from an ethnically and socially diverse country like India is not available on risk factors of ASD and existing studies from other countries have also proved to be inconclusive. There has been a huge focus on prenatal, perinatal and neonatal events as risk factors in various studies across the globe yet the results varied from one study to another. As there are no published reports on the prevalence of ASD, based on the studies available in other Asian countries regarding ASD prevalence (Kim et al., 2011; Sun & Allison, 2010) nearly 1.7 – 2 million individuals are estimated to be affected with ASD in India (Karande, 2006; Krishnamurthy, 2008). With such high estimated prevalence, it is important to assess the probable risk factors of ASD in Indian population as well.

Availability of standardized, proven research tools for such analysis is limited. Standardized, validated, and well planned questionnaires are one of the most important techniques to collect data about people, objects and events (Stawińska-Witoszyńska, Kowalska, Krzyżaniak, Krzywińska-Wiewiorowska, & Krzych, 2012). It lends credibility to design of epidemiological studies and helps researchers to reach relevant conclusions (Kazi & Khalid, 2012). Developing and validating questionnaires as an instrument for the epidemiological research on the risk factor of ASD and carrying out such a study from India would help in understanding the probable risks and its impact on development of the disorder. Thus, a comprehensive risk factor assessment questionnaire was developed to carry out epidemiological study from Indian population on the probable risk factors of ASD in India.

2.2. Study Design

2.2.1. Questionnaire Designing and Validation

The development of the questionnaire was done in a standardized manner, using an accepted measure development methodology which includes pilot testing, Criterion validity and reliability testing. The study was approved by the Institutional Human Ethics Committee (IHEC) and a written; informed consent was taken from all participants prior to the participation in the study. According to the regulations laid by the IHEC personally identifiable information, such as names, phone numbers and addresses, was also collected from participants with an assurance that the information will be stored confidentially in the database but will not be revealed.

2.2.2. Factor identification and questionnaire development

To start with, literature was searched for the reported Prenatal, Perinatal and Neonatal risk factors of ASD and the pertinent ones were carefully reviewed. The questionnaire was drafted by the principal investigator and presented to the research team. The research team analyzed the questionnaire drafted and modified the wording and content accordingly.

2.2.3. Participants and questionnaire validation

A total of 20 experts from the field of ASD research and statistics, and 70 parents – both parents of autistic children and parents of control children participated in the validation of the questionnaire.

The questionnaire was then pilot tested with 37 parents of the autistic children which was a convenient sample. Based on the pilot testing some more modifications were done to the questionnaire. A total of 43 items were listed in the questionnaire which had 36 questions with dichotomous answers in the form of Yes or No and 7 dealt with identifiable information

like age of the child, gender, disorder under spectrum diagnosed, diagnostic age, physician diagnosing, intervention services the kids were undergoing and name of the center where intervention is being taken. There was one question which had an open ended answer.

The questions were categorized into 1) Parental history which included Consanguinity, parental characteristics like age, number of scans, thyroid status, polycystic ovarian syndrome, gestational diabetes, Hypertension, Assisted reproductive technologies like Intra uterine injection, In vitro fertilization or hormonal interventions. 2) Fetal distress due to bleeding or amniotic fluid loss. 3) Labor complications due to cord around neck of the baby, pre – mature membranes rupture, Induced labor, Prolonged labor, Forceps labor, Vacuum induced labor, breech presentation of the baby. 4) Infections during pregnancy like Gastro – intestinal, Urinary tract and respiratory tract. 5) Medications taken for infections. 6) Neonatal characteristics like Child's thyroid status, Pre – term birth, Low birth weight, , Seizures, Hypoxia or lack of oxygen or birth asphyxia, Neonatal jaundice, Head injury before 28 days of birth, Pneumonia, Delayed birth cry. 7) Infant characteristics like constipation, Attention deficit hyperactivity disorder, Obsessive behaviour, Delayed milestones, Sleep disturbances, Food allergies, Abnormal gait or deviation from normal walking and Skin allergies like eczema etc.

2.3. Statistical evaluation

Statistical analysis of the questionnaire was done using SAS 9.1.3 (SAS Institute, Inc, Cary NC) version and Microsoft® Excel 2007. Both construct and reliability was tested where percentage agreement and content validity index were calculated to assess construct validity while internal consistency and test-retest validated the reliability of the questionnaire.

2.3.1. Construct validity

Construct validity refers to the degree to which the intended independent variable (construct) relates to the proxy independent variable (indicator) (Hunter & Schmidt 1990). Content evidence is often presented as a detailed description of steps to ensure that the items represent the construct (Haynes et al., 1995). The questionnaire finalized after pilot testing and modification was subjected to content and face validation. It was distributed among experts or scientists who have knowledge in the field of ASD and are into the research, those who are working with ASD children like Psychiatrists, Neurophysicians, Pediatricians, Psychologists and Special education providers like behavioral therapists, occupational therapists and speech therapists. The questionnaire was validated by parents of autistic children too. All the participants rated the questionnaire for relevance of the question with respect to the research, clarity of the wording, likelihood that the target audience would answer the question and the layout and style of the questionnaire.

2.3.1.1. Percentage agreement

Likert – type 10 rating scale (with 1 being least agreement and 10 being most probable agreement) (Preston & Colman 2000) was used to analyze the percent agreement. Percentage agreement was calculated to indicate the percentage of judges who agreed that the item was a good fit for the analysis. A percentage agreement $\geq$ 66% was used to indicate fair agreement (Portney & Watkins 2000). The formula used to calculate percent agreement (Araujo & Born 1985) is given below

$$Percent\ agreement = \frac{Number\ of\ participants\ rating \geq 6}{Total\ number\ of\ participants} \text{X } 100 \qquad [1]$$

2.3.1.2. Content Validity Index

The content validity was evaluated with the application of the Content Validity Index (CVI). A four point rating scale was provided to expert panel of 6 members for quantitative validation. The CVI indicates the proportion of experts who considered the item as content valid. The CVI is expressed as a percentage. For the quantitative analysis, content validity index (CVI) was also calculated. A value of 0.75 and above was considered as acceptable value (Lawshe 1975; Polit et al., 2007; Wynd et al., 2003). The observation of a CVI ≤ 0.75 implies the automatic review of this item, because it means that at least one of the judges did not approve its content validity. The items rated as "1" or "2" must be reviewed or eliminated (Lawshe 1975; Polit et al., 2007; Wynd et al., 2003). The formula used to assess content validity index of each item individually is represented below:

$$Content\ Validity\ Index = \frac{Number\ of\ responses\ as\ \text{“3”}\ or\ \text{“4”}}{Total\ number\ of\ responses} \text{ X } 100 \qquad [2]$$

2.3.2. Reliability Testing

Reliability refers to the reproducibility or consistency of scores from one assessment to another (Cook & Beckman 2006). Internal consistency reliability and test – retest reliability are the two commonly used estimators of reliability (Parsian 2009).

2.3.2.1. Internal consistency:

It denotes the extent to which the individual items that constitute a test correlate with one another or with the test total. One of the most widely-used indices of the internal consistency is Cronbach's α (Cronbach 1951) and the formula for which is as follows

$$\alpha = \frac{K}{K-1}\left\{1 - \frac{\sum_{i=0}^{n}\sigma_Y^2}{\sigma_X^2}\right\} \quad [3]$$

Where,

α - Cronbach's α (or Coefficient alpha).

K - Number of items constituting the instrument.

σ_x^2 - Variance of the summated scale score

$\sum \sigma_y^2$ - The sum of the variances of the individual items that constitute this scale.

The coefficient alpha is a general formula for scale reliability based on internal consistency. For the analysis of Internal consistency, the accepted value of Cronbach's alpha coefficient is greater than or equal to 0.6 (Cronbach 1951).

2.3.2.2. Test – retest

Test – retest procedure was used for reliability testing. In order to assess reliability of the questions, Kappa statistics, were applied. The formula employed for calculating Kappa (Cohen 1960) statistics is given below

$$\kappa = \frac{P_o - P_c}{1 - P_c} \quad [4]$$

Where,

Po – proportion of observed agreements

Pc – proportion of agreements expected by chance.

The interval between test and retest was 12 weeks. A total of 70 parents of cases and controls took the test and retest. Since the study is conducted around India, the parents participating in the validation study were from Chennai (3), Bangalore (4), Hyderabad (60) and Mumbai (3), who undertook the study twice for reliability testing. Both in the test and in the retest, the

compliance of the obtained answers “yes / yes”, “no / no”, “yes / no”, “no / yes” was expressed in absolute numbers and percentages. Cohen’s kappa values are given below:

- If < 0 was rated as poor;
- 0 – 0.20 as fair;
- 0.41 – 0.60 as moderate;
- 0.61 – 0.8 as substantial and
- 0.81 – 1 was considers almost perfect (Landis 1977).

Confidence intervals at $P = 0.05$ and concordance rates (%) were also calculated from test – retest procedures.

2.4. Questionnaire collection

2.4.1. Sampling data

Simple random sampling was done between September 2010 and December 2012 from individuals across 9 major Indian cities. The sample frame consisted of centres dealing with children with ASD for cases and schools with intellectually normal children in the case of controls. The centre selection was carried out based on the size, probability of finding children from various socio-economic backgrounds and to have a better distribution with wider presence across the country.

The sample size calculation for an expected ASD population size of around 2 million was found to be 385 (given $\alpha = 0.05$), and predicting a 20% refusal rate for participation in the study, more than 600 participants were approached and 500 cases were enrolled. An equal number of controls were recruited to achieve a case: control ratio of 1:1.

2.4.2. Data Sources

In India, an estimated 26 million children are born every year, of which about 10 million (42%) go unregistered (UNICEF, 2006). A single point of data collection through registered structured records was not possible because even today, in rural areas, births occur at home rather than at hospitals or primary health care centers, contributing to unregistered cases. Lack of public awareness and no demand for civil registration documents (birth certificates) is also one of the biggest challenges and the reason for low levels of registration. Even for those registered, registration is usually done by the local body – The Municipal Corporation, and the birth certificate issued includes only name of the child, place and time of birth. Sometimes the place of birth of the child and the place where the child is brought up might differ due to cultural practices leading to decreased monitoring of the health conditions thereafter. So, various independent organizations were approached for data collection across India to carry out our study.

2.4.3. Case ascertainment and enrolment

The information about major centers where children with ASD are enrolled was obtained from The National trust – Government of India, Psychiatric departments of various hospitals across 8 major cities (Hyderabad, Chennai, Mumbai, Bangalore, New Delhi, Mysore, Ahmedabad, Guntur), institutes like National Institute for the Mentally Handicapped (NIMH), various autism centers and by a thorough internet search. Collaborations were established with 70 centers across 9 cities for data collection which covered various hospitals, autism clinics, autism schools, special schools, therapy clinics. These included Government run or recognized centers and Institutes, large Non- governmental organizations (NGOs) where children from various lower economic backgrounds enroll due to the lower costs involved in therapies and other smaller centers, therapy clinics, large and small schools

dealing with children with ASD. These centers were preferred as they maintain direct and sustained contact with the families and individuals with ASD (Daley, et al., 2013).

Our study included children between 2 – 10 years of age across India. The criterion for age selection is based on the fact that the first signs of the disorder are normally seen even before the child is 3 years old (Mefford et al., 2012; Boyd et al., 2010). Concrete diagnosis is usually available between 3-4 years of age, though certain conditions like the Asperger's Syndrome may have a delayed diagnosis at around 5-6 years (CDC, 2008). Many children with behavioural abnormalities reported either by parents, relatives, general practitioners, psychologists, and schools are referred to specialists in child psychiatry for further evaluation and diagnosis. The psychiatrists are well trained to use the internationally accepted classification systems such as DSM IV and ICD 10 (Daley & Sigman, 2002). Apart from those two, ISAA - Indian Scale for Assessment of ASD is also used by psychiatrists across the nation (Patra et al., 2011) for diagnosis and giving certification. Childhood Autism Rating Scale (CARS) is also used commonly to classify case status (Rellini et al., 2008). Upon diagnosis, a report is provided to the parent regarding the ASD evaluation. Children with ASD, as reported by the parent and confirmed by the diagnostic report constituted our study sample. Within each centre, the response rate of the participating parents was 95% and the non – respondents were usually parents of those children who were not born in India or who were above 10 years of age, or because of their unwillingness to participate due to personal perceptions. Children under a suspicion for ASD but with no formal diagnosis, children with Cerebral Palsy and Down syndrome, were excluded from the study.

2.4.4. Enrolment of control subjects

The control populations (age and gender matched) were identified randomly across 9 major cities in India between September 2010 and December 2012. The enrolment was done

parallel to the recruitment of children with ASD. All the children were typically developing according to parental and teacher reports, and did not have any history of learning or psychiatric disabilities. Data was collected by establishing collaborations with schools (regular, government run and private school) as these represented all sections of the society from rural to urban from low socio-economic status to high socio-economic status (Muralidharan & Kremer, 2006). Also, data was collected by visiting houses randomly across the cities. Most of the children enrolled attended regular government and private schools and were progressing well as per teacher and parent's report.

2.4.5. Data collection

An informed consent was taken from parents of the participating children (both cases and controls) prior to the study. The various methods followed for data collection include:

1. ***Direct interaction:*** We directly interacted with parents (literate and illiterate), explained the questionnaire, took informed consent and collected the data. This constituted major part of the data collection.
2. ***By Trained staff:*** Teachers of special schools and clinics were educated thoroughly about the questionnaire, the wording and the relevance. They helped the parents to fill the questionnaire by translating the terms into the local dialect and by explaining the doubts regarding the questions. The parents themselves filled the questionnaire or the teachers filled the questionnaire on behalf of the parent. 40% of the parents took the questionnaires to home, checked the medical records available with them and filled the responses.
3. ***Telephonic interview:*** Since the questionnaire dealt with dichotomous answers for comorbid conditions, for those cases where the parents had reported "yes" for comorbid conditions, subsequently a telephonic interview was scheduled and information was collected for detailed account of those comorbid conditions like existence of food related

issues, description of typical behaviors with hyperactivity like impulsivity, distractibility or inattentive behaviors etc. Telephonic call was made to the parent by the researcher to fix appointment to collect the information. Parents were briefed about the study again to help them recollect the research, its purpose and the need for the collecting some more details of the reported comorbid condition.

Detailed explanation of questionnaire as given above coupled with parent's cooperation ensured minimum missing data. Though the age group under consideration was in range of 2 – 10, 70% of the children (cases and controls) were below 7 years of age minimizing the bias due to memory recall. The quality control of our questionnaire based study was ensured by comparing the data collected from questionnaire with the medical records available for a convenient percentage of study population (~20%) and was found to show 100% consistency.

2.5. Results and Discussion

2.5.1. Construct Validity

Additional content was not suggested by any of the 40 persons in the questionnaire development cohort. Most of the participants understood and scored well in the questionnaire but there were discrepancies in the clarity of the wording pattern for certain questions indicating a need for significant rewording of the questionnaire.

2.5.1.1. Percentage agreement

Percentage agreement was also calculated for each item for construct validation (Table 2.1). A total of 40 participants (20 experts in the field of Autism Spectrum Disorder research and statistics; 20 parents – both parents of autistic children and parents of control children) were approached for the validity testing. Each of the participants scored on a 10 point scale for each question. From our results (Table 2.1) it is clear that percentage agreement for all

questions was above 80%. As any percentage agreement $\geq$ 66% indicates fair agreement (Portney & Watkins, 2000), the validity of the questionnaire, with respect to the relevance of the content, clarity in wording, probability that the target audience is likely to answer the question and its layout is proved.

2.5.1.2. Content validity index (CVI)

An acceptable CVI in this study was determined to be 0.75 or above with 6 experts in the ASD and epidemiology field who were part of the panel (Haynes, Richard, & Kubany, 1995; J. Hunter & Schmidt, 2004; Preston & Colman, 2000). The CVI for each item was calculated (Table 2.1). Out of the 44 questions validated, the CVI for 42 questions were calculated to be 83.3 and above. The CVI of 2 questions pertaining to abnormal gait and head injuries before 28 days from birth was calculated to be 66.6%. With reference to the literature a value of 0.75 and above was considered as acceptable value while calculating CVI (Lawshe, 1975; Polit, Beck, & Owen, 2007; Wynd, Schmidt, & Schaefer, 2003). As the percentage of agreement for 95.4% questions was more than 80%, all the questions were included for their content.

2.5.2. Reliability Testing

2.5.2.1. Internal Consistency

The Cronbach's alpha was obtained using the total of 70 participants answering the questionnaire (cases and control). Cronbach's alpha coefficient value greater than or equal to 0.620 is accepted for Internal consistency (Cronbach, 1951). In our analysis, the alpha value (Table 2.1) ranged from 0.9 – 0.92 indicating a good repeatability of our designed questionnaire.

Table 2.1. Construct Validation by percent agreement and Content validity index, Internal consistency analysis by Cronbach's alpha.

Questions	Construct Validity testing		Reliability testing
	[a]Percent agreement (%)	[b]Content validity index (%)	[C]Cronbach's alpha coefficient
History			
child's age at diagnosis	100	83.3	0.91
Father's age at child birth	100	100	0.91
Maternal Characteristics			
Mother's age at child birth	100	83.3	0.91
Consanguinity among parents	100	100	0.91
Scans underwent by mother	100	83.3	0.91
Hyperthyroidism in mother during pregnancy	98	83.3	0.91
Hypothyroidism in mother during pregnancy	93	100	0.91
Polycystic ovarian syndrome in mother	100	100	0.91
Gestational diabetes in mother	100	83.3	0.91
Hypertension/high blood pressure in mother	98	83.3	0.92
Assisted reproductive technology			
Intra Uterine Injections taken by mother	100	83.3	0.91
InVitro fertilization taken by mother	100	83.3	0.91
Hormonal Medications taken by mother	100	83.3	0.91
Hormonal Injections taken by mother	100	83.3	0.91
Fetal distress			
Bleeding during pregnancy	100	100	0.91
Amniotic fluid loss during pregnancy	95	83.3	0.92
Labor complications			
Cord around neck of the baby during delivery	100	83.3	0.92
Pre matured membrane rupture during pregnancy	100	83.3	0.91
Induced labor	100	100	0.91
Prolonged labor	98	100	0.91
Forceps delivery	100	83.3	0.91
Vacuum delivery	95	83.3	0.92

Breech presentation of the child	100	83.3	0.91
Maternal infections			
Gastro - Intestinal Infections in mother during pregnancy	98	83.3	0.91
Respiratory Infections in mother during pregnancy	100	83.3	0.92
Urinary tract Infections in mother during pregnancy	88	83.3	0.92
Medications for any infections taken during pregnancy by mother	100	83.3	0.91
Neonatal events			
Child's thyroid status	100	83.3	0.91
Pre-term birth of the child	100	83.3	0.92
Low - birth weight of the child	100	83.3	0.91
Allergies like eczema or any skin allergy in child	100	83.3	0.91
Seizures in child	98	83.3	0.91
Birth asphyxia/hypoxia/low oxygen immediately after birth of child	98	83.3	0.91
Neonatal jaundice of child	98	83.3	0.91
Head injury before 28 days of birth of child	98	66.6	0.91
Pneumonia in child	100	83.3	0.91
Absence birth cry	100	83.3	0.91
Infant characteristics			
Constipation in child	100	83.3	0.91
Attention Deficit Hyperactivity Disorder in child	100	83.3	0.91
Obsessive behaviour in child	98	83.3	0.92
Delayed milestones in child	98	83.3	0.91
Sleep Disturbances in child	100	83.3	0.91
Food related allergies in child	100	83.3	0.91
Abnormal gait/ abnormal walk of the child	100	66.6	0.91

[a]Percent agreement – greater that 66% are significant values; [b]Content validity index - ≥ 0.75 acceptable CVI values; [C]Cronbach's alpha coefficient – ≥ 0.6 accepted values

2.5.2.2. Test – retest

Repeatability of the questionnaire was assessed using a validation procedure. The validation procedure was applied to a total of thirty eight questions on prenatal, perinatal and neonatal risk factors of ASD. More than 80% of the respondents answered all the questions. The kappa statistic (Table 2.2), for most of the parental characteristics was calculated to be 1 except for details regarding number of scans (less than or equal to 3 or more than 3) which gave a concordance rate of 98.5% and 97.1% with kappa statistics of 0.94 and 0.91. One question on consanguinity gave a concordance rate of 98.6% with kappa statistic of 1. In the fetal distress category, bleeding during pregnancy had a concordance rate of 98.5% with kappa statistics of 1, while for amniotic fluid loss the concordance rate was 100% with a kappa statistics of 1. All causal factors of labor complications and maternal infections during pregnancy had a concordance rate of 100% with kappa statistics of 1. One question regarding the medicines taken for any infections during pregnancy had a concordance rate of 98.5 with a kappa statistics of 1. In the neonatal factors, 8 questions (pre – term birth, low birth weight, seizures, birth asphyxia, neonatal jaundice, head injury before 28 days of birth, pneumonia, and delayed birth cry) had a concordance rate of 100% with kappa statistics of 1. While concordance rates of the answers to the questions regarding thyroid test status of the child was 98.5% (kappa - 0.94), constipation was 97.1 (kappa – 0.93), attention deficit hyperactivity disorder was 93.9 (kappa – 0.87), obsessive behaviour was 98.4 (kappa – 0.94), delayed milestones was 95.5 (kappa – 0.91), sleep disturbances was 98.6 (kappa – 0.97), food allergies was 99.5 (kappa – 0.87), abnormal gait was 98.5 (kappa – 0.94) and skin allergies was 98.5 (kappa – 0.90). In our study altogether, out of the 41 questions which had dichotomous answers, 27 questions had concordance rates of 100%, whereas the concordance rates were 93% for the rest 14 questions. According to the literature Cohen's kappa values between 0.81 – 1 considered to be almost perfect (Cohen, 1960; Landis

& Koch, 1977). With our values falling in the range, it is evident that our questionnaire has a good repeatability.

Table 2.2 Calculation of test – retest reliability by concordance rates and Kappa Statistics

Questions	Concordance Rate (%)	+Kappa statistic	CI*
History			
Consanguinity among parents	98.6	1	1.00-1.00
Maternal Characteristics			
Less than or equal to 3 Scans of mother	98.5	0.94	0.83-1.00
Extra scans of mother	97.1	0.91	0.80-1.00
Hyperthyroidism in mother during pregnancy	100	1	1.00-1.00
Hypothyroidism in mother during pregnancy	100	1	1.00-1.00
Polycystic ovarian in mother during pregnancy	100	1	1.00-1.00
Gestational diabetes in mother	100	1	1.00-1.00
Hypertension/high blood pressure in mother	100	1	1.00-1.00
Assisted reproductive technology			
Intra Uterine Injections taken by mother	100	1	1.00-1.00
Intra Uterine Injections taken by mother	100	1	1.00-1.00
Hormonal Medications taken by mother	100	1	1.00-1.00
Hormonal Medications taken by mother	100	1	1.00-1.00
Fetal distress			
Bleeding during pregnancy	98.5	1	1.00-1.00
Amniotic fluid loss during pregnancy	100	1	1.00-1.00
Labor complications			
Cord around neck of the baby during delivery	100	1	1.00-1.00
Pre matured membrane rupture during pregnancy	100	1	1.00-1.00
Induced labor	100	1	1.00-1.00
Prolonged labor	100	1	1.00-1.00
Forecep labor	100	1	1.00-1.00
Vacuum labor	100	1	1.00-1.00

Breech presentation of the child	100	1	1.00-1.00
Maternal infections			
Gastro - Intestinal Infections in mother during pregnancy	100	1	1.00-1.00
Respiratory Infections in mother during pregnancy	100	1	1.00-1.00
Urinary tract Infections in mother during pregnancy	100	1	1.00-1.00
Medications for any infections taken during pregnancy by mother	98.5	1	1.00-1.00
Neonatal events			
Child's thyroid test	98.5	0.94	0.83-1.00
Pre-term birth of the child	100	1	1.00-1.00
Low birth weight of the child	100	1	1.00-1.00
Allergies like eczema or any skin allergy in child	98.5	0.90	0.70-1.00
Seizures in child	100	1	1.00-1.00
Birth asphyxia/hypoxia/low oxygen immediately after birth of child	100	1	1.00-1.00
Neonatal jaundice of child	100	1	1.00-1.00
Head injury before 28 days of birth of child	100	1	1.00-1.00
Pneumonia in child	100	1	1.00-1.00
Absence of birth cry	100	1	1.00-1.00
Infant characteristics			
Constipation in child	97.1	0.93	0.84-1.00
ADHD	93.9	0.87	0.76-0.99
Delayed milestones in child	95.5	0.91	0.81-1.00
Sleep Disturbances in child	98.6	0.97	0.91-1.00
Food related allergies in child	99.5	0.87	0.74-1.00
Abnormal gait/ abnormal walk of the child	98.5	0.94	0.83-1.00

[+]Kappa statistic – Cohen's kappa - < 0 poor; 0 – 0.20 fair; 0.41 – 0.60 moderate; 0.61 – 0.8 substantial; 0.81 – 1 almost perfect

** Confidence Interval at $p \leq 0.05$*

2.6. Conclusion

The integrity of any research depends on the accuracy of the measures used, especially when exploring complex disorder like ASD. The results of the performed research demonstrated very good validity and repeatability for most of the analysed questions. This fact proves that the use of our questionnaire as a psychometric tool for the assessment of prenatal, perinatal and neonatal risk factors of ASD is proper and justified.

CHAPTER 3

PARENTAL RISK FACTORS OF ASD

Parental factors described in question no. 1 and 2 of the questionnaire are described in this chapter. With genetics and hormonal disturbances playing a very important role in the aetiology of ASD (V Guinchat & Thorsen, 2012; Hvidtjørn et al., 2011; Lehti et al., 2013; Lyall et al., 2011; Lyall, Pauls, Spiegelman, Santangelo, & Ascherio, 2012; Maimburg & Vaeth, 2007; Morrow et al., 2008; Zachor & Ben Itzchak, 2011), the evaluation of parental factors for the risk of ASD becomes necessary. Our study focused on parental age, consanguinity among parents, maternal thyroid malfunction, polycystic ovarian disease and maternal hormonal interventions.

Advanced parental age and consanguinity are reported to be involved in genetic disorders (Anello et al., 2009; Bittles, 2008). Advanced parental age is also reported to induce hormonal disturbances (Anello et al., 2009). Also, economic burden and requirement of an individual to adapt to changing cultural and political scenarios is increasing mental stress among Indian women which in turn is affecting hormonal levels, menstrual cycle and leading to infertility (Harris & Carey, 2000; Manlove, 2011). This has led to an increased use of various infertility treatments across the country which involves regulation of the maternal hormonal environment (Glud, Kjaer, Troisi, & Brinton, 1998). With genetics and hormonal levels playing an important role in the aetiology of ASD, it is important to explore parental factors for the risk of ASD.

In this chapter we describe five parental risk factors. Section **3.1** deals with literature review on *consanguinity as risk factor* followed by results and discussion. Section **3.2** describes *Polycystic Ovarian disease* as risk factor for ASD. Section **3.3** is regarding *thyroid hormone malfunction* as risk factor. Section **3.4** is about *maternal hormonal interventions* as a risk factor for ASD and Section **3.5** describes *parental age* as risk factor for ASD.

Statistical Analysis

A total of 500 cases were recruited for our study after confirmed diagnosis along with equal number of controls. Data regarding risk factors was collected from parents across 9 cities in India using a validated questionnaire. Statistical analysis of all risk factors was done using SAS 9.1.3 (SAS Institute, Inc, Cary NC) version. To explore the collective effect of several variables simultaneously, logistic regression was used to calculate Odds ratios and 95% confidence intervals (CIs) at 5% α. Frequency of occurrence of risk factor in cases and controls dictates the analysis pattern used. Only if the frequency of occurrence is at least 5, multivariable analysis was carried out. In all other conditions only univariable analysis was performed by using Fisher's exact test. A conditional logistic regression by adjusting gender, maternal age and child's birth year was performed for variables with frequency at least 5 as well as for categorical variables such as birth weight and gestation term. The model accuracy for the adjusted analysis was calculated to be $R^2 = 0.651$, which indicated it to be a good model.

3.1. Consanguinity and its association with Autism Spectrum Disorder

Marriages in India involve religious sentiments (Nath, Patil, & Naik, 2004). Despite changing mind sets where marriage between close relations (consanguinity) is stigmatised, it is still practised in many places of the world (L Jaber, Halpern, & Shohat, 1998). Consanguineous marriage involves marriage between individuals with a common ancestor where individuals prefer to marry within their clan (Morgan, 1870). The global prevalence of consanguinity is 20% with an estimated 8.5% children born to consanguineous parents (Modell & Darr, 2002). Consanguinity is practised in North and sub-Saharan Africa, Middle East, and West, Central, and South Asia, which are usually associated with low socio-economic status, illiteracy and rural residence (Bittles, Mason, Greene, & Rao, 1991). Weddings within clan, tribe or caste is also well established tradition in these countries and hence there exist a possibility that couples who regard themselves as unrelated may also exhibit high levels of homozygosity (Bittles & Black, 2010).

The consanguineous marriages are more prevalent among Arab Muslims and Dravidian Hindus (Rao, Asha, Sambamurthy, & Rao, 2009) of South India i.e., Kerala, Karnataka, Tamil Nadu and Andhra Pradesh (Kuntla, 2013). In India, the main reasons for consanguineous marriages are stronger family connections and the integrity of property (T. S. Rao et al., 2009). While considering the relationships, among Arab Muslim, union between first cousins are preferred while, in south Indian-Dravidian Hindu population, cross cousins as well as matrilateral cross-cousins marriages are favoured (Rao et al., 2009). Various studies consistently implicate a risk of medical complications associated with consanguineous marriages like increased risk of adverse perinatal outcomes including stillbirths (Hussain et al., 2001; Khoury & Massad, 2000; Stoltenberg, 1999), low birth weight (LBW) (Benson, 2005; Hussain et al., 2001; Jordan, 2007; Khalid et al., 2006; Khoury & Massad, 2000; Mumtaz et al., 2007; Sezik et al., 2006; Stoltenberg, 1999), preterm delivery (Al-Eissa &

Ba'Aqeel, 1994; Mumtaz et al., 2010), apnoea of prematurity (Tamim et al., 2003), infant and child mortality (Bittles & Black, 2010), congenital birth defects and malformations (Lutfi Jaber et al., 2005). Apart from these, the risk of an autosomal recessive disorder for a progeny of a consanguineous union is also well characterized (Bittles, 2008).

Autism Spectrum Disorder (ASD) has multifactorial inheritance (V Guinchat & Thorsen, 2012; Morrow et al., 2008). High susceptibility genes are implicated to play a significant role in the expression of a heterogeneous complex disease like ASD, and if such genes are rare and have autosomal recessive manner of transmission then consanguinity could be a contributing factor. Thus, it is important to evaluate the association of consanguinity with ASD in India. *A detailed methodology for questionnaire designing and data collection is provided in chapter 2.*

We evaluated the nature of consanguinity in our population to assess its role in ASD aetiology. Among ASD cases, (Table 3.1) (Fig. 3.1) 100 (20%) subjects reported consanguinity, of which 68 (13.6%) were first cousins, 15 (3%) were uncle-niece, 10 (2%) double first cousins and 7 (1.4%) second cousins. Among the first cousins, 13 (2.6%) were parallel cousins and 55 (11%) were cross cousins. In controls, 7.2% subjects reported consanguinity, of which 4.6% were first cousins, 1.4% uncle-niece relationships, 0.4% double first cousins and 0.8% second cousins. Among controls, first cousin breakup accounted for 1.8% parallel and 2.8% cross cousins.

The average level of inbreeding was assessed in terms of coefficient of inbreeding values for each population. For first cousins it was 0.0625, Uncle – Niece was 0.125, Double first cousins it was 0.125 and for Second cousins the coefficient of inbreeding was 0.0156 (Table 3.1). The ASD cases had significant level of consanguinity when compared to controls and statistical evaluation substantiated that there is a 3.2 times of risk (95% CI – 2.15, 4.82, *P* - <0.0001) for ASD when parents are consanguineous.

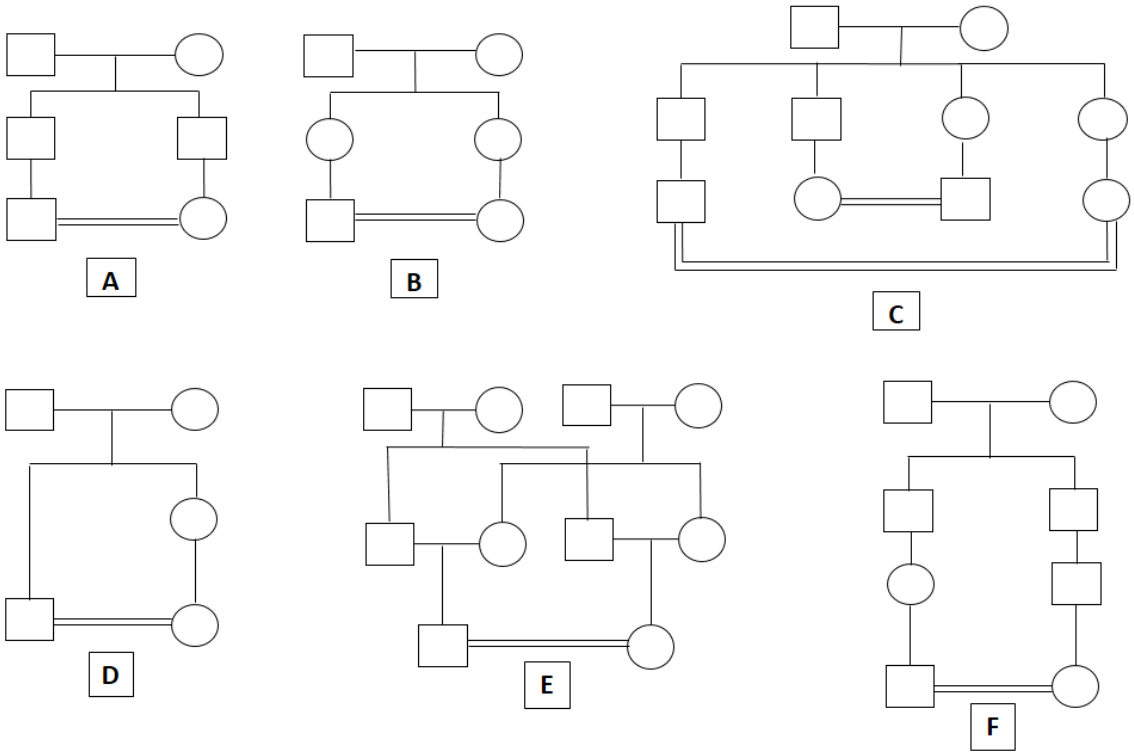


Fig. 3.1 Types of consanguinity in Indian Population. **A&B)** Parallel First cousins **C)** Cross First cousins. **D)** Uncle-niece **E)** Double First cousins **F)** Second cousins.

Table 3.1 Analysis of different types of consanguinity and inbreeding coefficients

Types of consanguinity	Number				Inbreeding coefficient
	Cases	%	Control	%	
Total	100	20	36	7.2	
First cousins	68	13.6	23	4.6	0.0625
Parallel Cousins	13	2.6	9	1.8	
Cross Cousins	55	11	14	2.8	
Uncle niece	15	3	7	1.4	0.125
Double first cousins	10	2	2	0.4	0.125
Second Cousins	7	1.4	4	0.8	0.0156

Among the factors analysed (Table 3.2), advanced maternal age [odds ratio of 1.58 (95% CI – 1.15, 2.15, *P* – 0.003)], consanguinity [odds ratio of 3.20 (95% CI – 2.15, 4.82, $P < 0.0001$)], gestational respiratory tract infections [odds ratio of 4.77 (95% CI – 1.80, 12.65, $P < 0.001$)], fetal distress [odds ratio of 3.11 (95% CI – 1.78, 5.44, $P < 0.0001$)], labor complications [odds ratio of 5.13 (95% CI – 2.98, 8.84, $P < 0.0001$)], and preterm birth [odds ratio of 1.83 (95% CI – 1.16, 2.88, $P < 0.008$)] remained significant in our study population. Also, LBW [odds ratio of 2.02 (95% CI – 1.39, 2.93, $P < 0.01$)], which was insignificant in our earlier study, became significant in this study. But when we conditioned consanguinity in multivariate analysis, all factors remained significant with decreased odds ratio (Table 3.2), and LBW again became insignificant (Table 3.2).

As per our study, parental consanguineous marriages had clear contribution towards the risk of ASD. Presence and absence of ASD was the sole criteria discriminating for the recruitment of cases and controls, irrespective of the prior knowledge about consanguinity status. Hence, there is no bias in recruitment of study population.

Table 3.2 Logistic regression of risk factors of ASD

Factors*	Cases		Controls		Unadjusted analysis		Adjusted analysis[#]	
	Number	%	Number	%	Odds ratio [95% Confidence Interval]	*P* - value	Odds ratio [95% Confidence Interval]	*P* - value
Advanced Paternal age[a]	340	68	334	66.8	1.05 [0.81, 1.37]	0.68	1.07 [1.05, 6.79]	0.98
Advanced Maternal age[b$]	121	24.2	84	16.8	1.58 [1.15, 2.15]	0.003	1.22 [0.96, 9.81]	0.01[+]
Consanguinity	100	20	36	7.2	3.20 [2.15, 4.82]	<0.0001	-	-
Respiratory tract Infections	23	4.6	5	0.1	4.77 [1.80, 12.65]	0.001	3.33 [2.09, 10.63]	0.03[+]
Fetal Distress	75	15	19	3.8	3.11 [1.78, 5.44]	<0.0001[+]	1.28 [1.00, 3.77]	0.004[+]
Labor complications	94	18.8	18	3.6	5.13 [2.98, 8.84]	<0.0001[+]	3.45 [1.70, 9.76]	0.002[+]
Pre-term Birth[c]	73	14.6	37	7.4	1.83 [1.16, 2.88]	0.008[+]	1.006 [0.19, 3.44]	0.05[+]
Low birth weight[d]	90	18	49	9.8	2.02 [1.39, 2.93]	0.01	1.02 [0.41, 4.20]	0.06

[+] *Significant values and 5% α;*

[#] *Adjusted for gender, child's age and maternal age*

[a] *Advanced paternal age = ≥ 30 years;*

[b] *Advanced maternal age = ≥ 30 years*

[c] *Preterm birth = < 37 weeks;*

[d] *Low birth weight = < 2, 500g*

The 3.2 times risk of consanguinity for the ASD aetiology in our study population had several important implications. It raises the possibility of recessively inherited genetic risk factors for the aetiology of ASD. Consanguinity is reported to have serious effects on fetal growth and development and increased the risk of congenital malformations (Kulkarni & Kurian, 1990). Moreover, it has been reported that children born to consanguineous parents had lower cognitive ability (M Afzal, 1988) and social behaviour (Md Afzal & Sinha, 1983) which are the major issues with ASD children. Hence, it is palpable that consanguinity could contribute towards the risk for ASD.

Consanguinity is also reported to increase the risk of adverse perinatal outcomes including stillbirth (Hussain et al., 2001; Khoury & Massad, 2000; Stoltenberg, 1999), low birth weight [LBW] (Benson, 2005; Hussain et al., 2001; Jordan, 2007; Khalid et al., 2006; Khoury & Massad, 2000; Mumtaz et al., 2007; Sezik et al., 2006; Stoltenberg, 1999), preterm delivery (Al-Eissa & Ba'Aqeel, 1994; Mumtaz et al., 2010), apnoea of prematurity (Tamim et al., 2003), infant and child mortality (Bittles & Black, 2010), congenital birth defects and malformations (Lutfi Jaber et al., 2005).

Analysis of LBW as a risk factor for ASD in our previous study (Mamidala et al., 2013) revealed that it is statistically not a significant contributor for ASD, either independently (P – 0.56) or with other pre-, peri- and neonatal risk factors (P – 0.60). However, inclusion of consanguinity in the multivariate analysis as in the present study, showed low birth weight to be a significant risk factor for ASD with an odds ratio of 2.02, indicating increased association with ASD. But, when we conditioned consanguinity, it again became insignificant suggesting that presence of consanguinity increases the risk of low birth weight in our population. This explains the link of consanguinity, low birth weight and ASD.

Preterm birth in our earlier study from India was a significant risk contributor for ASD (Mamidala et al., 2013). This remained significant in the multivariate analysis that

included consanguinity and several other variables (Odds ratio – 1.83). Thus, preterm birth remains a significant risk factor for ASD independently and with consanguinity.

The respiratory tract infections, fetal distress and labor complications are reported risk factors for ASD in India (Mamidala et al., 2013). Evaluation of these factors by conditioning consanguinity also established them to be significant risk factors for ASD.

In conclusion, our study demonstrates consanguinity as a significant risk factor for ASD. Our study highlights the necessity for preconception and premarital genetic counselling and recommends that the patrons of long-held ancient tradition of consanguineous marriages make informed choices in future. Our epidemiological approach in this highly inbred Indian population needs to be corroborated with genetic studies.

3.2. Thyroid Malfunction as risk factor for Autism Spectrum Disorder

Thyroid hormones, both T3 and T4 play a very important role in human reproductive health. Thyroid hormones are involved in the secretion/ action of ovarian steroids like Human Chorionic Gonadotropin (hCG), Luteinizing Hormone (LH) or Follicle Stimulating Hormone (FSH) and also interact indirectly with Sex Hormone Binding Globulin (SHBG) (Krassas, Poppe, & Glinoer, 2013). Also the maturation of oocytes demands normal levels of thyroid hormone (Jones, Hannan, Kaitu'u, Zhang, & Salamonsen, 2013). Apart from causing maternal reproductive impairments, thyroid hormone deficiencies during critical periods of brain development both *in utero* and in early post-partum period is well recognized cause of brain damage, leading to mental retardation, decreased intellectual capacities, psychomotor delay and deafness (Ahmed, El-Gareib, El-bakry, Abd El-Tawab, & Ahmed, 2008; Shils, Olson, & Shike, 1994). Maternal thyroid hormone deficiency like hypothyroidism in pregnant women have been reported to adversely affect their children's neurodevelopment which was proved from the subsequent performance on neuropsychological tests (Haddow & Palomaki, 1999)and thus affecting behavioural and emotional problems of the child later in life (Ghassabian et al., 2011). Hence looking at maternal thyroid malfunction as a risk factor for ASD seems to be logical. *A detailed methodology for questionnaire designing and data collection is provided in chapter 2.*

A total of 26 (5.2 %) (Table 3.3) mothers reported to have thyroid malfunction (11 had hyperthyroidism and 15 had hypothyroidism) during pregnancy. Similarly among the controls, there were 12 (2.4%) cases of thyroid malfunction (6 had hyperthyroidism and 6 had hypothyroidism) during conception.

Table 3.3 Percentage of mothers with thyroid malfunction

	Hyperthyroidism (%)	Hypothyroidism (%)	Total
ASD	2.2	3.0	5.2
Controls	1.2	1.2	2.4

Also, out of the 26 mothers of autistic children reporting thyroid malfunction, 3 mothers underwent hormonal treatment for infertility. However, the 95% CI around each odds ratio (Table 3.4) was wide and did not support any statistically significant difference in risk.

Table 3.4 Analysis of maternal Thyroid malfunction for the aetiology of ASD

	Unadjusted analysis			#Adjusted analysis		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***P***
Hyperthyroidism	2.26	0.78 - 6.56	0.13	2.35	0.81 - 6.85	0.11
Hypothyroidism	2.20	0.89 - 5.45	0.08	2.18	0.87 - 5.40	0.09

OR – Odds Ratio; CI – Confidence Interval; P – Probability

The interaction between thyroid hormones and nervous systems has long been apparent in clinical medicine (Chan & Kilby, 2000). Thyroid hormone levels were found to be critical signals for brain development. The presence of sufficient levels of maternal thyroid hormones during the first 10-12 weeks of gestation is important due to non-availability of fetal thyroid hormones at this stage. The fetal thyroid hormones production starts only after the first trimester (Morreale de Escobar, 2004). Deficiency of thyroid hormones during these critical periods of brain development, both in utero and in early postpartum period is a well-recognized cause of brain damage leading to mental retardation, decreased intellectual

capacity, psychomotor delay and deafness (Román, Reis, Defer, & Prockop, 2007). Hence, it is anticipated that maternal thyroid malfunction during pregnancy could have significant impact on fetal brain development. However, due to equal number of affected mothers in both cases and control population, it was not possible to establish a statistically relevant association of thyroid malfunctions as a risk factor for ASD. Sub-clinical abnormalities of maternal thyroid status which goes undetected (McLeod & McIntyre, 2010) followed by the period of diagnosis and treatment could play a pivotal role as a potential risk factor for ASD. Hence, more detailed analysis of subclinical thyroid conditions, detection and management of thyroid malfunction in mothers needs to be done, before rejecting this condition as a risk factor for ASD.

3.3. Polycystic Ovarian Syndrome and Maternal hormonal interventions as a risk factor for Autism Spectrum Disorder

India is a multifaceted society with various regional, religious, social and economic groups. Even today, bearing a child still remains an important factor in the socio-economic wellbeing of most Indian women (M. Das Gupta, Chen, & Krishnan, 1998). According to National Family Health Survey-3 (NFHS-3), fertility is on a decline in Indian population (NFHS-3, 2006) and World Health Organization (WHO) estimates the incidence of infertility to be in the range of 13% to 19% in India. Globalization and women empowerment has led to a change in the lifestyle of Indian women wherein there is integration of education, erratic work routines and household duties, leading to a stressful life (Banerjee, 2009b).

The stress is reported to affect women's menstrual cycle and hormonal make-up (Figà-Talamanca, 2006) and can exacerbate conditions like Polycystic Ovarian Syndrome (PCOS) (Manlove, 2011). Balancing new and traditional customs can add stress to young women of reproductive age with regard to fertility status (Harris & Carey, 2000; Manlove, 2011) and PCOS is also reported to be one of the factor for infertility among women (Schmid, Kirchengast, Vytiska-Binstorfer, & Huber, 2004; Teede, Deeks, & Moran, 2010). The key features of PCOS are ovarian dysfunction, polycystic ovarian morphology, and excessive androgen production (hyperandrogenism) and insulin resistance along with obesity (Allahbadia & Merchant, 2008; Ganie & Kalra, 2011; M. H. Hunter & Sterrett, 2000; Stefano Palomba et al., 2012). Mothers with PCOS during pregnancy are reported to have an *in utero* hyperandrogenic environment (Barry et al., 2010) which is also implicated to cause obstetric complications (S Palomba et al., 2010; Stefano Palomba et al., 2010).

This infertility has led to an increased use of various infertility treatments across the country. Infertility treatments involve administration of ovulation inducing drugs like Clomifene Citrate which is an estrogen receptor modulator, Human Menopausal

Gonadotropin **(**hMG) - which contains Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), Human Chorionic Gonadotropin (hCG) and Gonadotropin-Releasing Hormone Agonists (GnRH) (Glud et al., 1998). Also, natural or synthetic progesterone is administered to maintain the luteal phase. These hormones are administered depending on the cause of infertility and the protocol used (Glud et al., 1998). Thus, these treatments involve regulation of the maternal hormonal environment.

Maternal hormonal and nutrient environment has been implicated to impact the developing fetus, which in turn influences susceptibility to a wide range of neurodevelopmental, metabolic and psychiatric diseases in later life (Baron-Cohen, Lutchmaya, & Knickmeyer, 2004; Troisi et al., 2003; Zeltser & Leibel, 2011). Hormonal disturbances are also reported to have direct link to obstetric complications which is reported to influence the risk of ASD in children (Auyeung et al., 2009; Auyeung, Taylor, Hackett, & Baron-Cohen, 2010; Baron-Cohen et al., 2011; Brimacombe, Ming, & Lamendola, 2007). Medical, obstetric and psychological events during pregnancy are reported to influence the health status of an infant at birth and are anticipated to be the determinant of long-term health and quality of life (O'Keane & Scott, 2005). Therefore, PCOS and maternal hormonal disturbances are hypothesized to affect the growth of developing fetus contributing to neurodevelopmental abnormalities like ASD.

ASD is thought to involve both heritable and environmental component in its aetiology (Newschaffer et al., 2007). Environmental risk factors are implicated via complex gene-environment interactions, but no specific link between exposures and significant population effects are reported (Newschaffer et al., 2007). So, analysis of risk factors for their association with ASD could shed light on these links and help in exploring the pathways affected due to these risk aiding environments. The estimated increased prevalence coupled with the country's contemporary growth makes Indian population a compelling model for

such studies. Maternal conditions during gestation affect fetal development and can be a probable risk area which needs to be explored for their association with ASD. Thus, we aim to explore the association between hormonal interventions in mothers, with the aetiology of ASD in children. In a country like India, with large population, changing lifestyles and decreased fertility rates, it becomes utmost priority to look at the effect of maternal hormonal treatments on risk for ASD. *A detailed methodology for questionnaire designing and data collection is provided in chapter 2.*

Out of the 500 autism cases analysed, 40 mothers had PCOS (8%) while there were only 20 mothers among controls with PCOS (4%). This indicates a high proportion of PCOS among mothers of children with ASD. Logistic regression analysis (Table 3.6), further justified the findings that there is significant association of PCOS with ASDs (OR= 2.812 [CI: 1.532 – 5.159] *P*= 0.0008). Even after adjusting the variables to gender and child's birth year, the value for PCOS remained significant {(OR= 1.354 [CI: 0.942 – 4.230] *P*= 0.0513)}. This establishes a clear connection of PCOS as risk factor for ASD statistically.

Hormones like Estrogens, FSH, Progesterones and Gonadotropins are extensively administered during treatment of reproductive impairments (Brown, 2011). The mothers in our study group were administered the following treatment options:

a) Hormonal injections - Estrogens or Progesterones
b) Hormone oral drugs - Gonadotropins or hMG or Clomiphene or GnRH

Considering the total data, in the ASD population, 58 (12.3%) (Table 3.5) mothers underwent hormonal treatment regimens (23 [4.8%] had hormonal injections only, 19 [4%] had hormone oral drugs only and 16 [3.3%] in the form of both injections and oral drugs) for fertility. In the control population, a total of 21 (4.4%) (Table 3.5) mothers underwent hormonal treatment regimens (4 [0.8%] had hormonal injections only and 5 [1%] had hormone oral drug and 13 [2.7%] in the form of both injections and oral drugs) for fertility.

Out of the 58 mothers of children with ASD, 12 had undergone infertility treatment regimens due to polycystic ovary syndrome (PCOS) (Table 3.5). These results points out that the hormonal interventions were higher among mothers who have children with ASD than control population.

To further validate the results, logistic regression analysis (Table 3.6) was performed and the results indicated a significant association of maternal hormonal interventions with ASD (OR = 2.375 (CI = 1.396- 4.039) P = 0.0014). Even after adjusting the variables to child's birth year, gender and maternal age, the value of the maternal hormonal interventions remained significant {(OR = 2.240 (CI = 1.309 - 3.835) P = 0.0033)}.

Table 3.5 Percentage of mothers undergoing various kinds of hormonal treatment

	ASD		Control	
	N	%	N	%
Hormonal treatments	58	12.3	22	4.6
Hormonal Injections	23	4.8	4	0.8
Oral hormone drugs	19	4.0	5	1.0
Both injections and oral drugs of hormones	16	3.3	13	2.7
Hormonal treatment for clinical condition induced infertility				
PCOS mothers who underwent hormonal treatment for infertility	12	2.5	1	0.2

According to our hypothesis, we found that children born to mothers with PCOS had higher risk of developing ASD. There has been only one study analysing the association of PCOS in mother with ASD in children where in the researchers report the prevalence of pervasive developmental disorders among daughters born to mothers with PCOS (Stefano Palomba et

al., 2012). Apart from the above study, women with autism are reported to have higher rates of PCOS, male-pattern body hair growth, bisexuality or asexuality, delayed onset of menstrual cycle, irregular and painful menstrual cycle, severe acne, gender identity disorder, and family history of cancers of the breast, ovary, uterus and prostate (Ingudomnukul, Baron-Cohen, Wheelwright, & Knickmeyer, 2007; Ruta, Ingudomnukul, Taylor, Chakrabarti, & Baron-Cohen, 2011). While in another study, women with PCOS were reported to have an increased number of autistic traits (Hergüner, Harmancı, Hergüner, & Toy, 2012). Moreover, pre- and perinatal exposure to androgens are reported to be implicated in ASD aetiology (Auyeung et al., 2010; Baron-Cohen et al., 2011).

Maternal hormonal and nutrient environment is reported to impact the organisation and activation of brain (Bale et al., 2010; Fernandez-Twinn & Ozanne, 2010; Zeltser & Leibel, 2011), and any disturbances in the fetal environment could have consequences in the fetal development (Brown, 2011). Also, estrogen is administered as part of infertility treatments and estrogen exposure is already implicated in pathology of neurodevelopmental disorders like schizophrenia either from excessive dose, timing and/or duration of estrogen exposure or modification of estrogen receptor function (McEwen, 1987). Maternal reproductive hormone disturbances are reported to cause obstetric complications which have already been implicated as a risk for ASD (Newschaffer et al., 2007).

Apart from the above mentioned reports, limited studies are available for analysing the association of assisted reproduction in mother as a risk factor for ASD (Hvidtjørn et al., 2011; Lehti et al., 2013; Lyall et al., 2011, 2012; Maimburg & Vaeth, 2007; Zachor & Ben Itzchak, 2011). Results from our study in India is similar to other studies (Hvidtjørn et al., 2011; Maimburg & Vaeth, 2007) reporting an increased risk of ASD in children born to mothers who underwent hormonal treatment regimens. But since our results are based on

epidemiological study, quantitative assessment is required to further investigate the role of maternal hormonal interventions as a risk for ASD.

In conclusion, this epidemiological study from the Indian population sheds light on the association of maternal hormonal interventions to be a risk factor for ASD. Statistically, significant risk association was found for PCOS, hormonal interventions in mother and the aetiology of ASD in child, but more extensive future studies on quantitative assessment in terms of dosage, treatment regime and its outcome along with genetic predisposition are recommended.

Table 3.6 Analysis of PCOS and maternal hormonal treatment for the aetiology of ASD

	Cases		Controls		Unadjusted analysis				#Adjusted analysis			
	N	%	N	%	+OR	+95% CI		+ P	+OR	+95% CI		+ P
PCOS	40	8.0	20	4.0	2.812	1.532	5.159	0.0008*	1.354	0.942	4.230	0.0513*
Hormonal treatment	58	12.3	22	4.6	2.375	1.396	4.039	0.0014*	2.240	1.309	3.835	0.0033*

 # - The adjusted analysis was performed by adjusting child's birth year, gender and maternal age.

+ - OR – Odds Ratio; CI – Confidence Interval; P – Probability value

** - Significant value*

3.4. Parental age as risk factor for ASD

Evidence from epidemiological, genetic, and animal studies supports the hypothesis that advancing parental age, increases the risk of autism spectrum disorders (ASD) in their children (Lee & McGrath, 2015). Though many reports are available on the assessment of parental age as risk factor, individual studies are not wholly consistent in results: various studies have reported increased risk of ASD with both older fathers and older mothers; older fathers but not older mothers; older mothers but not older fathers; or, neither older fathers nor older mother (Lee & McGrath, 2015). The discrepancies in findings are likely influenced by issues such as sample size and characteristics, missing data, case ascertainment, and covariate adjustment. Hence, our study aims at understanding the risk of parental age towards the risk of ASD in a large population in India. *A detailed methodology for questionnaire designing and data collection is provided in chapter 2.*

Reference standards considered for analysis were 30 years and above for parental age (Grether, Anderson, Croen, Smith, & Windham, 2009). According to the statistical analysis, there was association found between advanced maternal age (OR: 1.80 [95% CI: 1.27, 2.54] P = 0.0008) and ASD (Table 3.7). Among the mothers of ASD cases 354 (75.1%) were below 30 years and 117 (24.8%) mothers were 30 years and above. While considering the control population, 397 (84.2%) mothers were below 30 years while 74 (15.7%) mothers were 30 years and above. With respect to paternal age analysis, 148 (31.4%) fathers were below 30 years and 323 (68.5%) fathers were 30 years and above among cases, while among controls 161 (34.1%) fathers were below 30 years and 310 (65.8%) fathers were 30 years and above.

Table 3.7 Univariable analysis of parental factors of ASD

	Cases		Controls		Odds ratio [95% Confidence Interval]	*P* - value
	Number	%	Number	%		
Parental factors						
Mother's age						
< 30	354	75.1	397	84.2		
≥ 30	117	24.8	74	15.7	1.80 [1.27, 2.54]	0.0008
Father's age						
< 30	148	31.4	161	34.1		
≥ 30	323	68.5	310	65.8	0.97 [0.72 , 1.30]	0.86

We analysed the association of parental age with ASD and found that advanced maternal age of 30 years and above to be significantly associated with ASD with the odds ratio 1.8 times higher for advanced maternal age compared to mother's who are below 30 years of age. This could be due to the fact that among women, advancement of age predisposes them to abnormalities in biological mechanisms, like alteration in hormonal factors affecting the in utero environment, epigenetic changes and nucleotide repeat instability etc (Anello et al., 2009). The synergistic effect of all these factors could affect fetal brain development leading to ASD (Anello et al., 2009; Durkin et al., 2008). Paternal age has received a lot of attention, with whole-genome sequencing studies linking older fathers to higher rates of de novo mutations and increased risk of ASD (Croen, Najjar, Fireman, & Grether, 2007; Lee & McGrath, 2015). But our study did not find any significant result even though earlier studies from other countries have shown association of paternal age with ASD (Burd, Severud, Kerbeshian, & Klug, 1999; Reichenberg et al., 2006; Shelton, Tancredi, & Hertz-Picciotto, 2010).

Advanced maternal age is directly linked to the risk of ASD. Since the advanced maternal age risk is linked to pregnancy complications, what would be the combined effect with prenatal, perinatal and neonatal risk factors? To explore this, multivariate analysis of all the parental, prenatal, perinatal and neonatal risk factors were carried out in chapter 4.

CHAPTER 4

PRENATAL, PERINATAL AND NEONATAL RISK FACTORS OF AUTISM SPECTRUM DISORDER

4.1. Introduction

The extreme complexity in the behavioral, developmental and associated medical conditions across ASD indicates existence of multiple unknown causal factors. The neuropathology of ASD remains unclear and the reported brain abnormalities among children with ASD indicate a probable link with disturbances in the *in utero* period (Gardener et al., 2011; Minshew & Williams, 2007; Pardo & Eberhart, 2007). Hence, it is imperative to focus of prenatal, perinatal and neonatal events as risk factors for ASD.

There has been a huge focus on pre- and perinatal events as risk factors in various studies across the globe, wherein disruptions and disorders of pregnancy, labor complications, fetal distress, low birth weight and premature birth have been studied and implicated in ASD (Bilder, Pinborough-Zimmerman, Miller, & McMahon, 2009; Bolton et al., 1997; Bryson, Smith, & Eastwood, 1988; Buchmayer et al., 2009; Burd et al., 1999; Burstyn, Sithole, & Zwaigenbaum, 2010; Cryan, Byrne, O'Donovan, & O'Callaghan, 1996; Deb, Prasad, Seth, & Eagles, 2007; Deykin, 1980; Dodds et al., 2011; Dubovický, 2010; El-Baz, Ismael, & El-Din, 2011; Finegan & Quarrington, 1979; Gardener et al., 2011; Ghaziuddin, Shakal, & Tsai, 2008; Gillberg & Gillberg, 1983; Glasson et al., 2004; Vincent Guinchat et al., 2012a; Hultman, Sparén, & Cnattingius, 2002; Johnson et al., 2010; Juul-Dam, Townsend, & Courchesne, 2001; Kolevzon, Gross, & Reichenberg, 2007; Kröger et al., 2011; Kuban et al., 2009; Larsson et al., 2005; Lord, Mulloy, Wendelboe, & Schopler, 1991; Losh, Esserman, Anckarsäter, Sullivan, & Lichtenstein, 2012; Maimburg & Vaeth, 2006; Mason-Brothers et al., 1987; Nelson, 1991; Ornitz, 1985, 1985; Piven et al., 1993; Schendel & Bhasin, 2008; Stein, Weizman, Ring, & Barak, 2006; Wallace, Anderson, & Dubrow, 2008; Zambrino, Balottin, Bettaglio, Gerardo, & Lanzi, 1995; Zwaigenbaum et al., 2002). Despite several studies being conducted worldwide to analyse the risk factors of ASD, the results are not conclusive.

Research reports on epidemiology of ASD from India are not available (Sharan, 2006). Analysis of such risk factors is pertinent in India, as the above mentioned risk conditions like fetal distress inducing conditions and labor complications are well documented to be prevalent with significant impact on survival and development of children in India (Kumar & Vishnu Bhat, 1996; March of Dimes, 2012; NFHS-3, 2006; Uma, Nisha, & Shikha, 2007). However, the exact impact of these conditions as risk factors for ASD needs to be established. Moreover, globalization, secondary to industrialization and the enhanced communication pathways, had led to significant cultural, political and economic changes, requiring an individual to adapt to these changing scenarios (Banerjee, 2009). This need for adaptation contributes to increased mental stress among individuals for gaining resources to cope (Banerjee, 2009). Mental stress, in turn, has reflected in stressful pregnancies due to associated psychosocial stress among Indian women (UNICEF, 2006). Hence, it is imperative to understand the implications of these on the pre, peri events and disease aetiology from India.

Thus, the aim of our study is to perform a population based cohort study to characterize the pre-, peri- and neonatal risk factors and assess their association with ASD in Indian population. Our study is the first epidemiological report from India and the results obtained strengthens similar observations from other studies reporting pre-, peri- and neonatal risk factors of ASD. Description of method is explained in methodology paragraph.

4.2. Results and Discussion

This is the first study to analyse the prenatal, perinatal and neonatal risk factors of ASD in Indian population. We conducted a questionnaire based case-control epidemiological study. The strength of the current study lies in its large country wide data collection with validated questionnaire, precise confirmation of the ASD diagnosis by physicians, active participation

of parents and volunteers (teachers and therapists) from various schools and hospitals in filling up of the questionnaire, personalized monitoring of collection, meticulous data analysis and also structured attempts to minimise the missing data.

The reference standards considered for analysis were less than 37 weeks for Abnormal gestational term and less than 2, 500 g for low birth weight category respectively (UNICEF, 2004; Muthayya, 2009). The model accuracy for the adjusted analysis was calculated to be $R^2 = 0.651$, which indicate it to be a good model.

4.2.1. Prenatal Characteristics

Six maternal conditions during gestation were analysed for their association with ASD by unadjusted analysis. Out of them clinical conditions like hypertension (1.69% in cases and 0.2% in controls) and gestational infections like respiratory tract infections (4% in cases and 1.0% in controls), were found to be significant with odds ratio of 1.8 (Table 4.1). Fetal distress is induced by many factors, understanding of the synergistic effect of all these factors seems logical. The occurrence of fetal distress was higher among cases when compared to controls (23.7% in cases and 4.2% in controls), and the logistic regression analysis (Table 4.1) presented it to be significant with an odds ratio of 5.50 ([95% CI – 2.44, 1.40] *P*-<0.0001). On the other hand, gestational diabetes, gastro – intestinal infections and urinary tract infections did not show significant association with ASD (Table 4.1) due to low number of controls.

Of the three gestational infections like respiratory, gastro – intestinal and urinary tract infections, we found the association of respiratory tract infection with ASD with an odds ratio of 3.8. The immune responses of the mother to these respiratory infections elicit the release of cytokines, which can cross the trans-placental barrier and can modulate neural function, survival, apoptosis, and expression of transmitters and neurotrophins in developing brain

(Depino, 2006). Cytokines also affect neural cell proliferation and differentiation, impairments of which are known to be associated with ASD (Ashwood et al., 2011a).

Table 4.1 Univariable analysis of parental and prenatal factors of ASD

	Cases		Controls		Odds ratio [95% Confidence Interval]	*P* – value
	Number	%	Number	%		
Parental factors						
Mother's age						
< 30	354	75.1	397	84.2		
≥ 30	117	24.8	74	15.7	1.80 [1.27, 2.54]	0.0008[a]
Father's age						
< 30	148	31.4	161	34.1		
≥ 30	323	68.5	310	65.8	0.97 [0.72 , 1.30]	0.86
Prenatal factors						
Gestational diabetes	5	1	4	0.8	0.71 [0.34, 4.79]	1.27[a]
Hypertension	8	1.69	1	0.2	7.93 [0.98, 63.74]	0.05[a]
Fetal distress	112	23.7	20	4.2	5.50 [2.44, 12.40]	<0.0001
Amniotic fluid loss	27	5.7	2	0.4	10.16 [2.34, 43.94]	0.001[a]
Bleeding	19	4	8	1.6	1.11 [0.40, 3.05]	0.83
Suboptimal intrauterine conditions	66	13.9	10	2.1	6.18 [2.99, 12.78]	<0.0001
Infections during pregnancy						
Gastro – intestinal	16	3.3	9	1.9	1.77 [0.77, 4.08]	0.17
Respiratory tract	19	4	5	1.0	4.79 [1.61, 14.22]	0.004[a]
Urinary tract	9	1.9	3	0.6	3.05 [0.82, 11.35]	0.09[a]

[a] *Fisher's Exact test was used to obtain P values when frequency of occurrence was less than 5.*

4.2.2. Perinatal Characteristics

Two assisted delivery methods and five complications observed during labor were analyzed (Table 4.2). Co-occurrence of more than one labor complication is not uncommon, hence understanding the collective impact of labor complications as risk factor for ASD seems logical. The occurrence of labor complications were higher among cases when compared to controls (15% in cases and 2.3% in controls) and the logistic regression analysis presented it to be significant with an odds ratio of 5.48 ([95% CI – 2.99, 11.57] *P*- <0.0001) but univariable analysis of individual factor was not significant enough. Moreover, analyses of various assisted delivery methods were did not show any significant association with ASD.

Bleeding during gestation, amniotic fluid loss or any other suboptimal intrauterine conditions have been implicated to cause fetal distress (Kaur, Kaur, & Cantt, 2012). According to Indian National Family Health Survey – 3 (NFHS – 3) there is rise in vaginal bleeding among women from both urban and rural areas (NFHS-3, 2006). Vaginal bleeding has been found to be associated with high rate of fetal loss and adverse infant outcomes like prematurity, Intra Uterine Growth Retardation (IUGR), still birth and neonatal death (Karim, Bakhtawar, Butta, & Jalil, 1998; Sipila, Hartikainen-Sorri, Oja, & Wendt, 1992) indicating fetal distress. Fetal hypoxia is one of the manifestations of this fetal distress and has been reported to induce conditions like placental abruption, threatened premature delivery, emergency caesarean section, forceps delivery, spontaneous abortion to varying degrees of cerebral damage (Quinn, Kinney, Munir, Crowley, & Miller, 2008). Also, amniotic fluid loss which is generally termed as oligohydramnios has been implicated to cause pregnancy complications like ruptured membranes and placental insufficiency as well as congenital anomalies (Chhabra, Dargan, & Bawaskar, 2007). Such instances of stressful conditions during pregnancy are conceptualized to be on a rise in major metropolitan cities of India in the last decade (Dole, 2003). According to our study, fetal distress has been found

significantly associated with ASD and the odds ratio was estimated to be 5.13. Our findings are consistent with previous findings that fetal distress induced during pregnancy is associated with aetiology of ASD. Thus, any suboptimalities of the fetus during gestation as indicated above can have adverse effect on the development of the fetus.

Though our investigation identified hypertension to be a risk factor for ASD in univariable analysis, but we couldn't draw any conclusions due to low representation in the control group.

According to NFHS – 3, pregnancy complications are on rise among Indian women and so is the maternal mortality due to these complications (RGI, 2006). Obstructed and prolonged labor has been reported to cause asphyxia resulting in infant death or brain damage (Ashford, 2002). Also, nuchal cord has been reported to be a risk factor for mild, chronic, pre-labor fetal hypoxia (Hashimoto & Clapp, 2003) and is associated with a subclinical deficit in neurodevelopmental performance at 1 year of age (Clapp, Lopez, & Simonean, 1999). Premature – membrane rupture has been reported to be involved in causing fetal distress (Kaur et al., 2012) and moderate to severe neurodevelopmental impairments in infants (Spinillo et al., 1995) as well. Our study reports that more labor complications arising due to pre – matured membrane rupture or breech presentation of the child or Nuchal cord or induction of labor and prolonged labor and other labor or delivery complications were observed among mothers of children with ASD rather than in control population with an odds ratio of 4.52. Thus, complications occurring during labor affect the neurodevelopment of the fetus and infant in later stages and can contribute towards the risk of ASD.

4.2.3. Neonatal Characteristics

Neonatal risk factors included the birth weight of the child, gestational age, delayed birth cry, birth asphyxia, neonatal jaundice, seizures and eczema (Table 4.2). Among the factors

analysed, pre – term birth (14.1% in cases and 6.1% in controls), delayed birth cry (5.2% in cases and 1.2% in controls), birth asphyxia (11.2% in cases and 1.0% in controls), and neonatal jaundice (13.5% in cases and 3.6% in controls) were significantly associated with ASD and had an odds ratio of greater than1.1.

The analysis of neonatal factors gave significant results with respect to delayed birth cry, birth asphyxia, pre – term birth and Neonatal Jaundice with an odds ratio of greater than 1.1. In India, 50% of all child mortality is due to pre-term births and other associated complications (March of Dimes, 2012). Neonatal consequences of preterm delivery reported include developmental delay, hearing impairment and intraventricular hemorrhage (Marlow, Wolke, Bracewell, & Samara, 2005; Ward & Beachy, 2003; Wood, Marlow, Costeloe, Gibson, & Wilkinson, 2000). Also, an increased incidence of ADHD and other behavioural abnormalities have been observed among children born preterm (Bhutta, Cleves, Casey, Cradock, & Anand, 2002). Thus, pre-term birth seems to be a good risk factor candidate to be explored. Our finding shows that pre term birth of less than 37 weeks is significantly associated with ASD with odds ratio of 2.11, and is consistent with the other studies reporting association of preterm birth with ASD.

Our results are at par with other studies (Maimburg & Vaeth, 2006; Xin Zhang et al., 2010) reporting a link between neonatal jaundice and ASD with an odds ratio of 2.89. Neonatal jaundice is due to accumulated bilirubin (mostly conjugated) physiologically or pathologically, and has been implicated to cause damage to the central nervous system and also can lead to bilirubin encephalopathy (Maimburg & Vaeth, 2006; Xin Zhang et al., 2010). Delayed birth cry and birth asphyxia are also significantly associated with odds ratio of 2.68 and 10.63. Delayed birth cry and birth asphyxia are known to induce hypoxic conditions leading to neurological consequences also (Low, 2004; Nelson, 1991).

Table 4.2 Univariable analysis of perinatal and neonatal factors of ASD

	Cases		Controls		Odds ratio [95% Confidence Interval]	*P* - value
	Number	%	Number	%		
Perinatal factors						
Assisted delivery						
Forceps mediated delivery	7	1.4	1	0.2	3.76 [0.41, 34.17]	0.23[a]
Vacuum mediated delivery	3	0.6	1	0.2	1.66 [0.14, 18.97]	0.68[a]
Labor complications	71	15	11	2.3	5.48 [2.99, 11.57]	<0.0001
Induced labor	9	1.9	1	0.2	4.76 [0.55, 40.71]	0.15[a]
Prolonged labor	13	2.7	1	0.2	6.07 [0.73, 50.25]	0.09[a]
Pre - matured membrane rupture	1	0.2	1	0.2	0.41 [0.018, 9.69]	0.58[a]
Breech presentation of the child	5	1	1	0.2	2.26 [0.22, 23.21]	0.49[a]
Cord around neck of the child	8	1.6	1	0.2	1.95 [0.21, 17.96]	0.55[a]
Other complications of labor and delivery	35	7.4	6	1.2	6.30 [2.59, 15.28]	<0.0001
Neonatal factors						
Pre – term birth	67	14.1	29	6.1	2.11 [1.29, 3.42]	0.002
Low birth weight	64	13.5	45	9.5	1.13 [0.73, 1.76]	0.56
Delayed birth cry	25	5.2	6	1.2	3.22 [1.26, 8.25]	0.01
Birth asphyxia	53	11.2	5	1.0	11.15 [3.88, 32.01]	<0.0001[a]
Neonatal jaundice	64	13.5	17	3.6	3.58 [2.02, 6.35]	<0.0001
Seizures	11	2.3	1	0.2	6.23 [0.74, 51.87]	0.09[a]
Eczema	1	0.2	2	0.4	0.10 [0.004, 2.74]	0.17[a]

[a] *Fisher's Exact test was used to obtain P values when incidence was less than 5*

4.2.4. Adjusted analysis

Adjusted analysis (Table 4.3) with maternal age at gestation, gender and birth year of the child was also carried out for the factors with minimum frequency of 5 in both the case and control group for the calculation of adjusted odds ratio (aOR). The factors considered were parental age – father & mother; fetal distress, gestational infections – respiratory and gastro – intestinal, labor complications and neonatal factors – pre – term birth, low birth weight, birth asphyxia, neonatal jaundice, and delayed birth cry. The factors which were significant after adjusted analysis were mother's age at gestation (aOR – 1.59), fetal distress (aOR – 5.13), respiratory infections (aOR – 3.80), labor complications (aOR – 4.52), pre – term birth (aOR – 1.78), birth asphyxia (aOR – 10.63), neonatal jaundice (aOR – 2.89) and delayed birth cry (aOR – 2.68). Our results suggest that even after adjustment of gender, maternal age and birth year, the factors remained significant with odds ratios greater than 1.5, indicating true association, as was the case in univariable analysis.

Table 4.3 Adjusted analysis of risk factors of ASD

	aOR	95% Confidence Interval	*P* - value
Parental factors			
Father's age	1.05	[0.76, 1.46]	0.73
Mother's age	1.59	[1.09, 2.32]	0.01[a]**
Fetal distress	5.13	[3.03, 8.69]	<0.0001****
Gestational Infections			
Respiratory infection	3.80	[1.18, 12.29]	0.02**
Gastro intestinal infection	2.24	[0.54, 9.26]	0.26
Labor complications	4.52	[2.27, 9.01]	<0.0001****
Neonatal factors			
Pre – term birth	1.78	[1.07, 2.93]	0.02*
Low birth weight	1.13	[0.71, 1.79]	0.60
Birth asphyxia	10.63	[3.69, 30.59]	<0.0001****
Neonatal Jaundice	2.89	[1.58, 5.28]	0.0006****
Delayed birth cry	2.68	[0.99, 7.25]	0.05*

** Significant values*

Our study did not find any significant association of low birth weight (<2,500g) with ASD. Among neonatal factors, low birth weight and preterm birth are considered to be predictors of an adverse prenatal environment (Xin Zhang et al., 2010). Low birth weight has been reported to be a risk factor for psychiatric disorders like ADHD, anxiety symptoms etc… (Botting, Powls, Cooke, & Marlow, 1997; Hack, Klein, & Taylor, 1995). Surprisingly in the Indian population, unlike other studies low birth weight was not a major risk factor. This could be due to the fact that underweight children are not unusual even in the control population. Also, due to the exclusion criteria used where cases with cerebral palsy and intellectual disabilities were not included in the study where the frequencies of occurrence of low birth weight children are equally higher (Mervis, Decoufle, Murphy, & Yeargin-Allsopp, 1995).

Though this study gives comprehensive data from the Indian population, it is limited by certain constraints like vastness of the region with multiple ethnicities, no published report on incidence and prevalence, the non-availability of structured and reliable record keeping on maternal and fetal conditions in the country, dependence on maternal memory for data acquisition, etc. Thus, there is a need for further extension of this study to address these limitations. Nevertheless, many of our reports are consistent with other reports that prenatal, perinatal and neonatal environment plays a very important role in neurodevelopment and aetiology of ASD.

4.3. Conclusion

Our study categorically implicates many pre, peri and neonatal conditions to be risk factors for ASD independently and collectively, adding important country specific information to existing literature. Out of all the factors analysed, advanced maternal age, fetal distress and gestational respiratory infections were found to be associated with ASD and had an odds ratio

of 1.8. Evaluation of perinatal and Neonatal risk factors showed labor complications, pre-term birth, neonatal jaundice, delayed birth cry and birth asphyxia to be associated with ASD and had an odds ratio greater than 1.5. This study, being the first representation from a multi ethnic and multidimensional society like India becomes noteworthy. This adds impetus to the fact that additional focused investigations are necessary on physiological and genetic changes contributed by these risk factor inducing environments. Such a high number of risk factors being implicated in India also necessitate the true understanding of the actual incidence of ASD in this country.

CHAPTER 5

PARENTAL PERSPECTIVES OF COMORBID CHALLENGES IN ASD

5.1. Introduction

Autism Spectrum Disorder (ASD) is characterised by impairments in social interaction, communication, and restricted, repetitive and stereotyped patterns of behaviour, interests and activities (DSM-IV, 1994). Apart from typical symptoms observed among children with ASD, the condition is observed with the presence of other comorbid symptoms and disorders like Attention deficit/ hyperactivity, eating disorders, anxiety, depression, aggression, self-injury, and abnormal sleep patterns etc., which are not accounted by ASD diagnosis itself (Matson & Nebel-Schwalm, 2007; Srinath et al., 2005). It is often not realized that existence of comorbid conditions have a role in the occurrence of certain behavioural problems, attention, activity, and thought disturbances observed in a child with ASD (Leyfer et al., 2006). Thus, during counselling after ASD diagnosis is made, these conditions may not be given utmost preference and parents may or may not be anticipating these comorbid conditions at later stage. But, it is indeed necessary to educate parents about the probable coexistence of these conditions which may aggravate the ASD challenge and thus, help them select the treatment procedure to be opted for.

Every child diagnosed with ASD is different. With ASD being a combination of numerous different problems which vary with the individual, finding healthcare for an individual can often be very confusing and frustrating to the parent (Lovaas, 1987). Significant improvements in cognitive and behavioural functioning of autistic children were observed if the associated comorbid conditions are recognized early and managed (Stahmer, Collings, & Palinkas, 2005). Also, intervention intensity is highly dependent on the characteristics of the conditions upon which the family focuses and can be modified as per requirement, for e.g., some children may have predominant problems with sleep while others with proper sleep may be challenged with hyperactivity. This will also have implications on the monetary aspects involved in the preferred therapy as well.

ASD is a pervasive condition which requires a wide array of services like health and educational (T. Daley et al., 2013). The existence of comorbid conditions act as greater challenges and puts more pressure on the caretaker. Awareness about the existence of conditions which are not typically found in general checklist among professionals providing intervention services is important due to the difficulties faced by parents or caregivers in management of these conditions as well (Aluri & Karanth, 2002). This situation is further worsened due to the absence of enough intervention services and availability of service providers' across India, high costs involved per intervention session and availability of nominal funding services, concessions or benefits from the Government of India (A. Gupta & Singhal, 2005). Hence, education about the other associated conditions within the Indian ASD framework would help parents and professionals for early and ease of diagnosis as well as for designing effective, economical and combination of various therapy procedures.

Moreover, it is reported that due to the existence of cultural beliefs among Indian families there is a delay in early recognition of ASD symptoms. For example, the notion that Indian children speak single words by 1 to 1.5 years of age still exist among many parents, and so some Indian practitioners recommend that speech should not be considered delayed until the child is 3 years old (Daley & Sigman, 2002). Some of the Indian families accepted the abnormal behaviours of their child as a part of their family lives and did not consider it to be symptomatic of any particular disorder (Hackett, 2009). Indian parents have delayed symptom recognition like problematic behaviours than those reported in the western countries (Daley & Sigman, 2002). Thus, there is a need to educate parents and professionals about the existence of ASD associated comorbid conditions in the Indian context, apart from those observed in typical ASD cases.

Hence, this chapter deals with an epidemiological evaluation of existence of comorbid conditions among children with ASD in the Indian population. This study addresses only

those conditions which have link to behavioural issues like sleep disturbances, constipation, food allergies, skin allergies and hyperactivity. The present chapter addresses these issues. A detailed methodology is outlined in methodology section.

5.2. Results and Discussion

A sample of 500 children diagnosed with ASD was enrolled for the study. The male to female ratio observed was 5:1 (Table 5.1). Majority of the respondents answering the questionnaire were mothers and 90% of the children were below 7 years of age, which takes care of the memory bias limitation (Table 5.1).

The conditions reported among our study group are constipation, sleep disturbances, food and skin allergies and hyperactivity (Fig. 5.1) (Table 5.2). These conditions either existed independently or there was existence of 2 or more conditions together. 22% of parents reported that their child had constipation while 33.2% of the parents reported that their child had issues with sleep. Conditions were varying with 23.8% parents reporting that their child has food related allergies while 14% reported skin related allergies. Hyperactivity was the highest prevalent condition with 73.2 % of the parents found their child to be hyperactive.

Table 5.1 Details of the children and their parents for analysis of comorbid conditions

Characteristic		
	N	%
Age (years)		
2 – 3	254	50.8
4 – 6	227	45.4
7 – 10	19	3.8
Gender		
Male	421	84.2
Female	79	15.8
Respondent		
Mother	420	84
Father	60	12
Other relative	20	4

Table 5.2 Number of children with ASD with associated medical conditions

Condition		
	N	%
Constipation	110	22
Sleep Disturbances	166	33.2
Food allergy	119	23.8
Skin Allergy	70	14
Hyperactivity	366	73.2

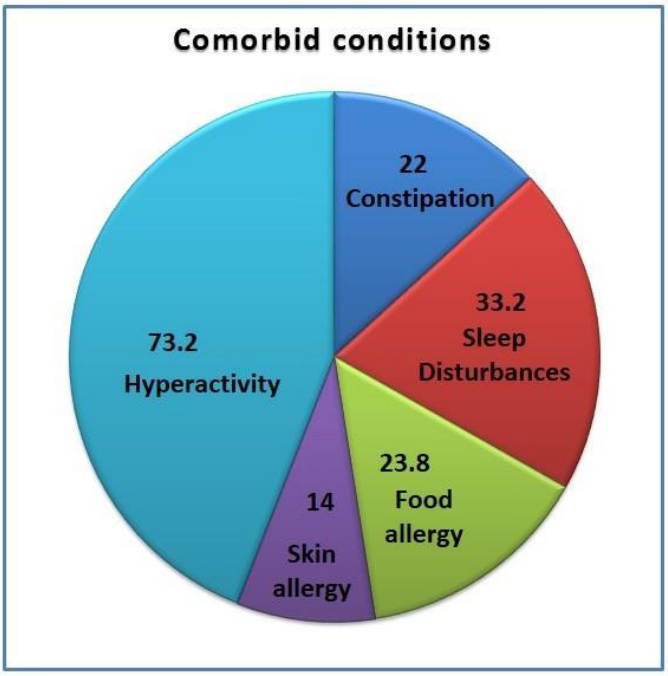


Fig. 5.1 Analysis of comorbid conditions in children with ASD

27.8% of mothers reported occurrence of hyperactivity along with sleep disturbances among children, while 4.2% mothers also reported the coexistence of food allergies with the above mentioned conditions (Fig. 5.2) (Table 5.3).

Table 5.3 Analysis of the existence of two or more conditions among children with ASD

Condition		
	N	**%**
Constipation & Sleep Disturbances & Hyperactivity	50	10
Constipation & Sleep Disturbances & Hyperactivity & food allergies	21	4.2
Sleep Disturbances & Hyperactivity	139	27.8

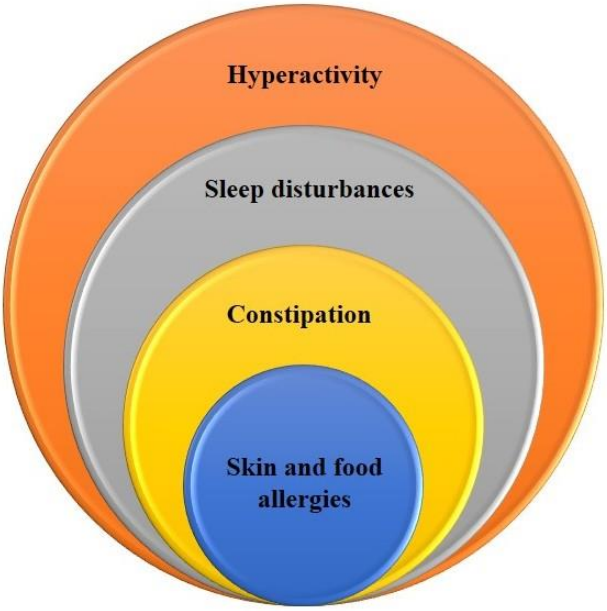


Fig. 5.2 Co-existence of comorbid conditions

Results from this large national survey highlight the health challenges of children with ASD and for their parents. As part of our study we discuss here about various comorbid conditions and the need to analyze them critically during diagnosis of ASD for providing better intervention service.

73.2% children with ASD in our population were reported to be hyperactive. Our findings are consistent with the findings from other studies reporting hyperactivity, impulsivity and inattention to be common among autistic children (Aman, 2004). This evaluation is important in India owing to the fact that due to the huge monetary aspects involved in ASD treatment, limited availability of funds for interventions from government side, which forces both parents to work (A. Gupta & Singhal, 2005) and it becomes much more difficult for the care takers when the child is hyperactive. Hence, this condition has to be taken care of. Moreover, with such high reporting of hyperactivity, it becomes necessary to look into the factors that might also contribute to hyperactivity typically, for e.g., Gastro-Intestinal (GI) problems (Buie et al., 2010) and food allergies (Gurney, McPheeters, & Davis, 2006), sleep disturbances etc. which are evaluated in our study as well. Moreover, in India,

where culture plays very important role in life, often abnormal behaviour of a child is accepted as normal and is part of their family lives (Hackett, 2009). Hence, more awareness to parents is needed regarding hyperactivity and the probable conditions aggravating the symptoms.

Eating disorders as well as problems with eating, food allergies (Billstedt, 2000; Gurney et al., 2006; Jyonouchi, 2009; A. Rao, Koch, Ghosh, & SureshKumar, 2010) and skin related allergies (Gurney et al., 2006) are also prevalent among children with ASD. This is also observed in our study where 14% of parents reported that their child had food and skin related allergic conditions. The presence of allergic conditions might aggravate the behavioural abnormalities in ASD (Gurney et al., 2006) which is one of the major issues while handling a child with ASD.

GI disorders are also reported to cause behavioural abnormalities (Buie et al., 2010) and parents report an improvement in certain behaviours by the resolution of GI symptoms with the help of dietary intervention measures, such as a casein-free/ gluten-free diet (Jyonouchi, 2010). Wheat is one of the major staple foods in India and also a very rich source of gluten. Excluding wheat from diet remains a challenge for these parents because of poor awareness and limited availability of gluten free food options (Verma, 2013). Even among health professionals knowledge about gluten free options in India is limited. Most of the gluten free recipes and literature available doesn't suit to the needs of Indian taste (Verma, 2013). Hence, gluten free diet might not be feasible for many families in India leading to aggravation of symptoms in some cases. This might be one of the reasons for high rates of hyperactivity rates in our study group. Thus, there is a need improvise the intervention services which would be usable by common man in India. More focused gluten free dietary options within the Indian context also needs to be explored and made available for parents of Indian children with ASD, suitable to their palate, culture and taste. Pre-available options

from the western countries cannot be unquestionably simulated due to their cost and differences that exists in perception of food across these cultures.

Apart from all the above mentioned conditions, 22% of parents report that children suffer with constipation problems. With reports of 5–10% children without autism having constipation our estimates indicate a necessity to diagnose such problems (Youssef & Lorenzo, 2001). The probable reasons for such high incidence could be the fact that children with combination of neurological and developmental deficits as in the case of ASD have toileting problems frequently (Dalrymple & Ruble, 1992). Moreover, reports from western countries suggest that effects of inborn neurodevelopmental psychiatric deficits including inborn GI abnormalities might be a reason for gut abnormalities like constipation and treatment of these in ASD cases are reported to show improvement in the conditions (Dalrymple & Ruble, 1992; Pang & Croaker, 2011). Thus, treatment of such conditions should also be considered while ASD diagnosis and treatment.

Report from other study group indicates a higher prevalence of sleep disturbances among children with ASD (Williams, LL, & A, 2004). Nevertheless, the frequency of parent - reported prevalence of sleep disturbances among the children with ASD in our study is low when compared to the prevalence rates in western countries reporting an incidence of 50 – 60% of sleep related disturbances among children with ASD (Williams et al., 2004). The differences in the sleep disturbances ratios could be due to differences in sleep practices which vary across cultures (De Gioia, 2009), co-sleeping which is common among Indian families (Bharti, Malhi, & Kashyap, 2006), sleep environment and different parental expectations of sleep (Ravikiran, Jagadeesh Kumar, & Latha, 2011). When considering the coexistence of sleep disorders with hyperactivity, 27.8% of the parents confirmed the coexistence. Thus, these conditions necessitate behavioural intervention programs addressing hyperactivity and its coexistence with sleep difficulties among children with ASD.

The findings from our study would create awareness among professionals as well as parents regarding the co–existence of other conditions which are not typical in the ASD checklist. This is important so that treatment procedures are initiated for these comorbid conditions along with those which are typical observed among ASD children especially in cases where the child is just diagnosed with ASD. This would in turn, help in early diagnosis and in turn better intervention services. Educating the qualified service provider with the child's specific medical and psychological requirements would minimize the social and sensory issues of the child at new place. Thus, would in turn improve the effectiveness of the visit and the patient as well as parent satisfaction level.

The strength of our study lies in its large population size considered. The nature of the data in the study is a limitation as the parental report of associated conditions is not validated with medical reports. Over or under reporting of the symptoms by the parents of children with ASD is also anticipated, but our findings are consistent with the prevalence rates mentioned in another studies.

5.3. Conclusion

Our findings document the parent – reported prevailing conditions among children with ASD indicating a need for systematic diagnosis and assessment by the physicians as it has direct implication on the child and the families involved. This assessment also projects the need for therapies from economic, care-giving, and psychosocial perspectives. Likewise, an economic evaluation of health services related to ASD systems would be valuable to understand and plan for present and future resource needs, perhaps including special training of intervention providers with the perspective of the patients involved.

CHAPTER 6

EVALUATION OF THE EFFECT OF AYURVEDIC MEDICINES ON NEUROTRANSMITTER PRODUCTION IN IMR 32 CELL LINE

6.1. Brief review

Children with ASD show presence of comorbid conditions like Attention deficit/ hyperactivity, eating disorders, anxiety, depression, aggression, self-injury, abnormal sleep patterns etc. (Matson & Nebel-Schwalm, 2007) which often lead to certain behavioral problems (Leyfer et al., 2006). The aetiology of these behavioral problems is reported to involve neurotransmitters imbalances (Wang, Angley, Gerber, & Sorich, 2011).

Neurotransmitter is a substance that is released from the axon terminal of a presynaptic neuron on excitation, and travels across the synaptic cleft to either excite or inhibit the target cell (Lauder, 1993). Apart from their role in early neuronal development, neurotransmitters have been involved in a range of physiological and behavioral functions such as sleep, aggression/hyperactivity, sensory perception, appetite, cognition & attention, motor activity etc., which are often reported to be disrupted in ASD (Wang et al., 2011). For example, GABA levels are known to be decreased in anxiety (Durant et al., 2010) and its increase has sedative effect and promotes sleep. On the other hand, 5-HT or Serotonin is reported to be involved in a broad spectrum of behavioural, psychological processes like social behavior, aggression and anxiety as well as in various psychiatric disorders (Murphy et al., 2004). Similarly Dopamine (DA) is a neurochemical molecule responsible for brain's emotional response to an external influence. It also modulates processes and behaviors that are abnormal in individuals with ASDs including motor functions, social behavior, attentional skills and perception (Ernst et al., 1997; Hosenbocus & Chahal, 2012; Kindregan et al., 2015) and executive functioning, such as analyzing, planning and prioritizing (Hosenbocus & Chahal, 2012).

Neurotransmitters are also reported to play a prominent role in ASD risk. Decreased levels of GABA were observed in the brains of individuals with ASD (Gaetz et al., 2014; Harada et al., 2011; Rojas, Singel, Steinmetz, Hepburn, & Brown, 2014). While, 5-HT levels

are reported to be low in brain and also administration of Selective Serotonin Re-uptake Inhibitors (SSRI) increased extracellular serotonin availability thereby reducing the symptoms of irritability and rigid-compulsive behavior in individuals with ASD (Adamsen et al., 2014; Buitelaar & Willemsen-Swinkels, 2000; Veenstra-Vanderweele et al., 2009). Low DA levels were reported in children with ASD (Martineau, Barthélémy, Jouve, Muh, & Lelord, 1992) and the role of the DA in the ASD is clinically proven through the use of neuroleptics and DA-receptor blocking agents (Emanuele, 2015).

Treatment procedures for the behavioral problems include various intervention services like conventional allopathic treatment or by complementary & alternative therapies (CAM) and sometimes a combination of both therapies (Esbensen, Greenberg, Seltzer, & Aman, 2009). Ayurvedic medicine is one form of CAM that is used to restore the proper order and function in the body (Chopra & Doiphode, 2002). Perceptions of safe usage of alternate medicine i.e., a belief that there are fewer side effects and the opinion of it being natural, encourage the wider usage of alternate medicine by many families. This was also re affirmed in the response given by the parents to question No. 16 (dealing with type of medical interventions) of our questionnaire (Hanson et al., 2007).

Ayurveda is an age old tradition originating from India (Chopra & Doiphode, 2002). It is designed to promote good health and longevity of life. A part of ayurvedic treatment involves administration of various plant (herbal) extracts either individually or in combination for the treatment of illness (Chopra & Doiphode, 2002). Brahmi or *Bacopa monnieri* (BM) is one of the popular ayurvedic herbal medicines prescribed for CNS disorders to improve learning and memory (Vaidya, 1997). There are various medicines available which has Brahmi as major component. Brahmi vati (BV) is a poly – herbal mineral formulation consisting of Brahmi (*Bacopa monnieri* or BM) and another thirty six ingredients (Mishra, Mishra, Ghosh, & Jha, 2013). Brahmi ghrita consists of Brahmi (*Bacopa monneri*),

Vacha (*Acorus calamus*), Kushtha (*Sassurea lappa*), Shankhapushpi (*Convolvulos pluricalis*) and Purana Ghrita (Deo & Reddy, 2012). Saraswata ghrita is also a polyherbal drug consisting of majorly BM and Haridra (*Curcuma longa*), Amlaki (*Embellica officinalis*), Haritaki (*Terminalia chebula*), Pippali (*Piper longum*), Vidanga (*Embelia ribes*), Kushta (*Saussurea lappa*) and Vacha (*Acorus calamus*) (Ansari, Tripathi, & Ansari, 2013). These herbal medicines are generally prescribed for neurological and psychiatric disorders (Shinomol, Muralidhara, & Bharath, 2011). Also, BM is already known to improve behavioral alterations and oxidative markers in sodium valproate induced autism in rats (Sandhya, Sowjanya, & Veeresh, 2012).

ASD is reported to be associated with comorbid conditions leading to behavioral issues and research suggests the role of neurotransmitters imbalances in the aetiology of behavioral problems. Also, alterations in neurotransmitter levels are reported to be associated with ASD. With reports suggesting usage of alternate therapies for the treatment of behavioral issues and neurological disorders and also non-availability of scientific data to prove their exact action, our study aims at understanding the effect of these ayurvedic medicines on neurotransmitter levels using IMR 32 cell line.

6.2. Materials and Methods

6.2.1 Chemicals and Reagents

All reagents were of cell culture and analytical grade. Deionized as well as Milli-Q Water (MQW) (Elix 3, conductivity 0.12 µS cm^{-1}) were used in the preparation of solutions. Standard neurotransmitters like Serotonin, Dopamine, and Gamma Amino Butyric acid, HPLC grade acetonitrile, HPLC grade Methanol, Di-Potassium ethylene diamine tetra acetic acid (EDTA), DNAse - RNAse free water, Formic acid (HCOOH), Dulbecco's Minimal Essential Medium (DMEM), F-12 Ham, Fetal Bovine Serum, Penicillin, Streptomycin, were

procured from Sigma Aldrich. While, Phosphate Buffer Saline (PBS), Trypsin-EDTA, Di-methyl sulphoxide (DMSO) were procured from Himedia.

6.2.2. Cell culture

The human neuroblastoma cell line, IMR 32 was obtained from neuroblastoma tissue. The cells have a small neuroblast – like appearance (Carbone, Sher, & Clementi, 1990) and also have properties of tumor cells with a doubling time of 48 hours (Tumilowicz, Nichols, Cholon, & Greene, 1970). The IMR 32 cells are a typical N-type neuroblastoma cells, which tend to form clumps and they also poorly adhere to the culture dish (Rossino, Defilippi, Silengo, & Tarone, 1991). IMR 32 cells respond to retinoic acid by forming extended processes with typical growth cones (Rossino et al., 1991). Treated IMR 32 cells also expressed an up-regulation of integrin α1/β1 expression, a laminin and collagen receptor (Rossino et al., 1991).

The main reason for choosing IMR-32 for this research was because of its human origin, large size, when differentiated it mimics large projection neurons of the human cerebral cortex and has previously been used in studies to express and quantify neurotransmitters (Yusof, Neal, Aykin, & Ercal, 2000). We used liquid chromatography with mass spectroscopy to quantify the neurotransmitters from IMR 32.

6.2.2.1 Procurement and establishment of Cell-culture IMR-32

IMR 32 cell line was procured from National Centre for Cell culture (NCCS) Pune, India. IMR 32 was cultured in DMEM medium (Sigma Aldrich), 10% fetal calf serum (Sigma Aldrich), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Sigma Aldrich), in 37 ºC, humidified air with 5% CO_2. The medium was changed twice a week and cells were split at about 80% confluency.

6.2.2.2 Differentiation of Cell lines

Prior to differentiation, cells were grown in serum free medium for 3 days. To examine the effects of retinoic acid (or retinol) on cell growth, cells (10^5) were plated in 25-cm^2 flasks with 5 ml of culture medium at a density of $2x10^6$ cells per ml. To the 24 hours cultures, the medium was replaced with medium containing 10uM retinoic acid and the procedure was carried out for 7 days. The cultures were re-fed every fourth day with retinoic acid containing medium throughout the course of the experiment. Cells were detached with the trypsin/EDTA solution and counted with a hemocytometer. Cell viability was determined via trypan blue exclusion method.

6.2.2.3. Toxicity assay of the ayurvedic medicines

MTT assay was performed on the cell lines using various concentrations of methanolic extract of the ayurvedic medicine to determine the half maximal inhibitory concentration (IC_{50}) of the drug. All herbal formulations were serially diluted from 1mg/ml to 10ng/ml in medium. 100% methanol was the control. This medium was added to the differentiated neurons and tested for cytotoxicity using MTT assay. The IC_{50} of all herbal formulations were 50ug/ml.

6.2.2.4. Drug Treatment to IMR 32

To the differentiated IMR 32 cells plated in a 96 well plate at a density of 2 $x10^6$ cells per mL, 50ug/ml of each formulation in medium was added to each well. The experiment was performed in triplicates and negative control was cells with only medium without the herbal formulation. These cells were grown for 24 hrs. After 24 hrs they were trypsinized and pelleted and supernatant medium was stored at -80^0C till further use.

6.2.3. Ultra-fast liquid chromatography – electrospray ionization mass spectrometry (UFLC- ESI – MS)

Mass spectrometry (MS) is one of the fastest growing fields in analytical science today. MS allows for directly detecting neurochemicals lacking fluorescent or electroactive properties (Pitt, 2009). MS has the ability to do fast, structure-specific detection of molecule providing characteristic information like molecular weight and fragmentation ions (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). By far the most commonly used methods in neurochemical analysis are Electron Spray Ionization (ESI) and Matrix Assisted Laser Desorption /Ionization (MALDI) (Fenn et al., 1989). ESI is most widely used because it employs soft ionization, can operate at low flow rates, and can detect both small and large molecules (Fenn et al., 1989). ESI-MS is optimal for analytes that show high surface activity and are easily charged (Fenn et al., 1989) like monoamines and neuropeptides due to their primary amine or quaternary ammonium moiety (Enke, 1997; Zhou & Cook, 2000). It aids by providing information regarding their molecular weight and amino acid sequence. This allows compound identification by database screening (Aebersold & Mann, 2003). Taken together, MS is a versatile and powerful tool for studying the neurochemistry of brain processes encompassing the entire range of compounds involved in neural transmission. RP-HPLC in conjunction with MS is widely used in many bioanalytical fields because of the excellent stability, separation efficiency, and easily matched interfacing. The coupling between RP-HPLC and ESI-MS can provide low range detection limits, yielding a powerful capability that is particularly suitable for the detection of neurotransmitters (Ewing, Wang, Sheeley, & Sweedler, 2008). The fact that RP-HPLC allows large volumes to be loaded on the column further improves the detection capability for neurotransmitters.

6.2.3.1 Instrument

The liquid chromatography was performed using C18 column, 5µm particle size (Phenomenex, Luna, ODS, 150x4.6mm) equipped with a Photo Diode Array Detector (PDA). The mobile phase consisted of aqueous 0.1% formic acid with 15µg/ml Na_2-EDTA (A) and 0.1% formic acid in acetonitrile (B) introduced in the ratio of 92:8. The injection volume of each standard, the processed samples was 100µl and run time for each sample was less than 7 min.

The mass analysis of all the samples injected was done using UFLC-MS [LCMS-2020, Single Quadrapole Liquid Chromatograph Mass Spectrometer, SHIMADZU Corporation] in ESI mode with UV detection. The LCMS had a binary gradient elution pumping system, an inbuilt on-line degasser, UFLCXR autosampler and a diode array detector. All parameters were controlled by LabSolutions LCMS software, version 5.4 (SHIMADZU Corporation). The standards and the samples were introduced to the single quadrapole without splitting with a flow rate of 0.4mL/min into a spray interface and at a maximum pressure of 400 kgf/cm^2. The temperature of the heat block was set to 200°C and the column was maintained at 35°C. Nitrogen was used as nebulizing gas [1.5L/min] and the drying gas [15L/min].

6.2.3.2. Preparation of test drug solution and ghrita

All drugs were weighed and dissolved in methanol to get a stock concentration of 1mg/ml and stored in the refrigerator till further use. As ghrita is prepared in ghee, the medicinal part is extracted from it using separating funnel. Accurately weighed 5 gm formulations were extracted with equal amount of hexane and methanol (20 ml each) by means of separating funnel. It was shaken vigorously and allowed to stand for 5 min for separating the two layers. Methanolic layer was again treated with 10 ml hexane till it was free from fat. Hexane layers

were discarded. The volume was made up to 25 ml with methanol in a volumetric flask and filtered through 0.22 micron filter. This preparation was used as stock solution.

6.2.3.3. Sample preparation for MS

The stock solutions of Dopamine, Serotonin and GABA, were prepared at a concentration of 10 mM in autoclaved filtered MilliQ water and stored at -20°C. Depending on the compound, these stocks were diluted to 1mM, 100µM, 100nM and 1nM concentrations using autoclaved filtered MilliQ water to check for the sensitivity of detection of the compound at different concentrations by HPLC and LC-MS. 100µl of each sample was injected for analysis.

Frozen medium was thawed and filtered using 0.22 micron filters (nominal molecular mass cut-off: 10,000) and was subjected to centrifugation at 13,000 rpm for 22 min. 100ul aliquots of the filterate were used for LCMS.

6.2.3.4. Quantification validation

The calibration standards for the quantification validation included neurotransmitters DA, 5-HT, and GABA in the range of 10^{-5} mM – 1mM. Calibration plots were generated for seven concentrations of each neurotransmitter and a blank, by plotting the peak area (y) versus the standard concentration (x). Regression equations were calculated with a weighting factor 1/x. The linearity with regression coefficients (R2) was always greater than 0.99.

The limit of detection (LOD) which is the peak area of blank plus three times the standard deviation of the blank was calculated using the blanks and calibration plots. The limit of quantification (LOQ) which is the peak area of blank plus ten times the standard deviation of blank was also calculated (Carrera, Sabater, Vilanova, & Sogorb, 2007). The repeatability of the proposed method was ascertained by injecting six replicates of fixed concentration and finding out the peak area in percentage relative standard deviation (%RSD)

(Carrera et al., 2007). Intra-day and Inter-day repeatability was determined using standard solutions and samples containing the neurotransmitters in the range from 10 – 5 mM – 1mM. Accuracy was calculated as: 100 x (E−T)/T, where E is defined as the calculated concentration using the corresponding calibration curve and T is the nominal concentration of the standard sample (Carrera et al., 2007). Control cells were spiked with neurotransmitters and analyzed on three separate days to determine accuracy. The matrix effect of the column used could be assessed by standard addition, sample clean-up, post-column infusion, matrix-matched standards and internal standards (Kilcoyne & Fux, 2010). We used standard addition method wherein standard neurotransmitters were added to cell secretions of control samples and the signal of standard alone and that of spiked samples were compared. The concentration of both was maintained at 100ng/ml.

6.3. Results and discussion

Retinoic acid was used to differentiate IMR 32 (Fig. 6.1). All neurotransmitters of our interest were eluted within 7 minutes of the run. A typical chromatogram of standard neurotransmitter along with the LCMS analysis is shown in Fig. 6.2, where 5-HT and DA were detected at 280 nm with retention times 4.48 and 5.47, while, GABA was detected at 214 nm with a retention time of 3.76min. The conformation of the neurotransmitter presence was achieved by LCMS analysis (Fig. 6.3) where GABA was confirmed at a mass of 104, 5-HT at 177 and DA at 154. The stability and reproducibility of the standards and samples of neurotransmitters were monitored by intra-day and inter-day analyses. Also, linearity (Fig. 6.4), precision, accuracy, LOQ and the LOD were assessed for DA, 5-HT, and GABA and all results were satisfactory and within range (Table 6.1).

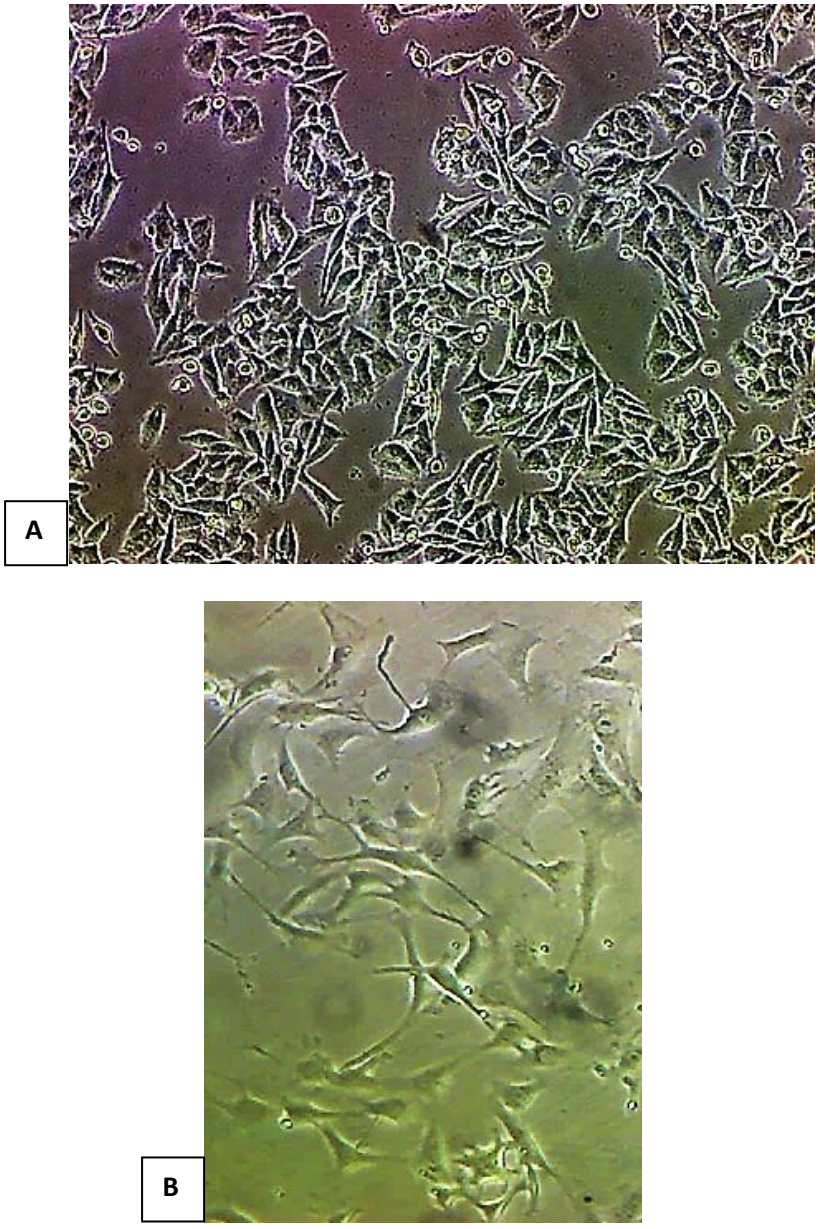


Fig. 6.1 Images showing A) undifferentiated IMR 32 and B) Differentiated IMR 32

Table 6.1 Quantification validation parameters of the developed LCMS method

Analyte	Retention time (min)	Regression equation	R^2	Detection limit (ng/mL)	Quantification limit (ng/mL)	Inter-day repeatability (n = 6, %RSD)	Intra-day repeatability (n = 6, %RSD)	Accuracy (%)
Dopamine	5.47	y = 2E+07x + 522823	0.9982	12.3	26	0.64	4.3	88.8 – 98.5
GABA	3.82	y = 4E+07x + 450975	0.9992	10.7	27.2	0.65	1.8	97.8 – 99.8
Serotonin	4.48	y=5E+07x + 6424.1	0.9989	10	29	0.45	2.5	97.7 – 99.3

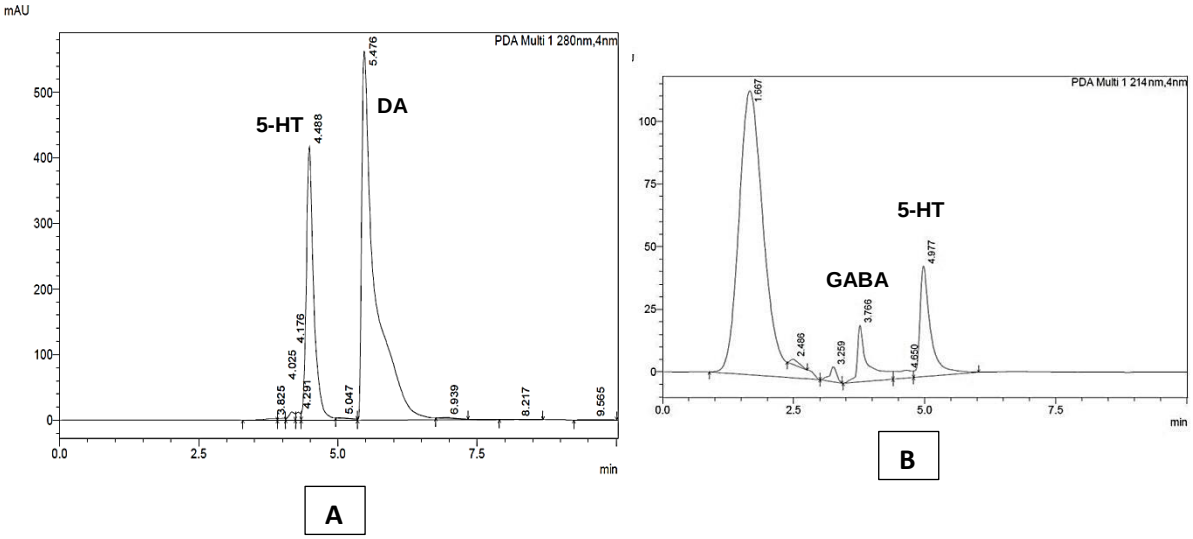


Fig 6.2 A) PDA analysis of standard neurotransmitters at 280nm, 5-HT – 4.48; DA – 5.47 **B)** PDA analysis of neurotransmitter standards at 214nm GABA – 3.76 retention

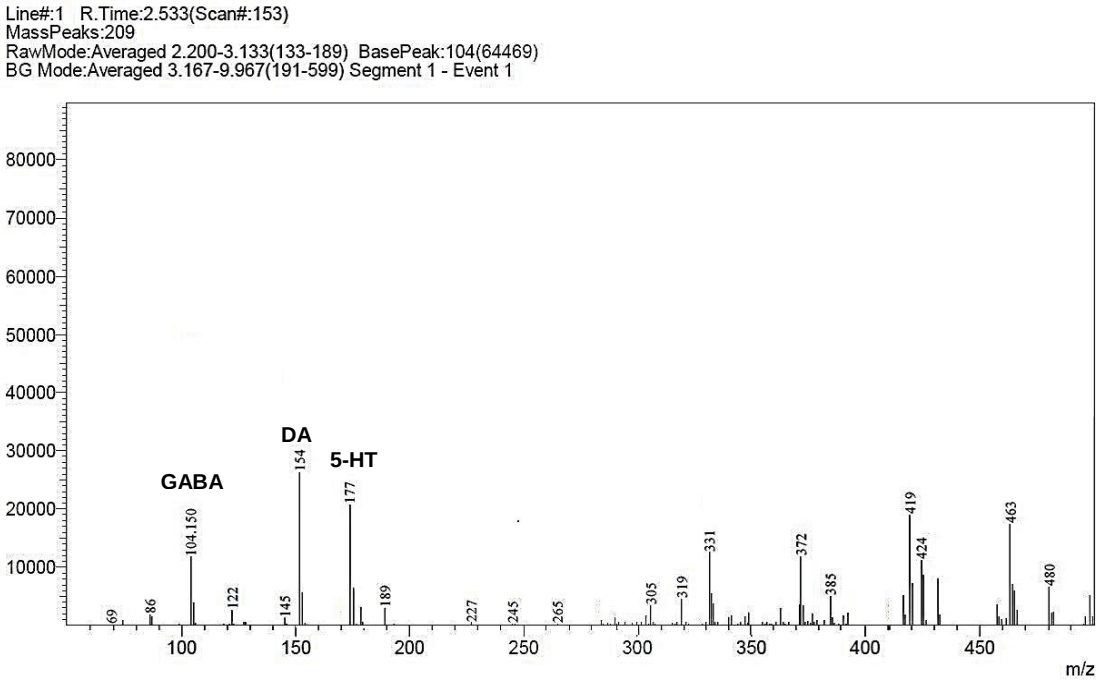


Fig. 6.3 LCMS analysis of standard neurotransmitters. GABA – 104; DA – 154; 5-HT - 177

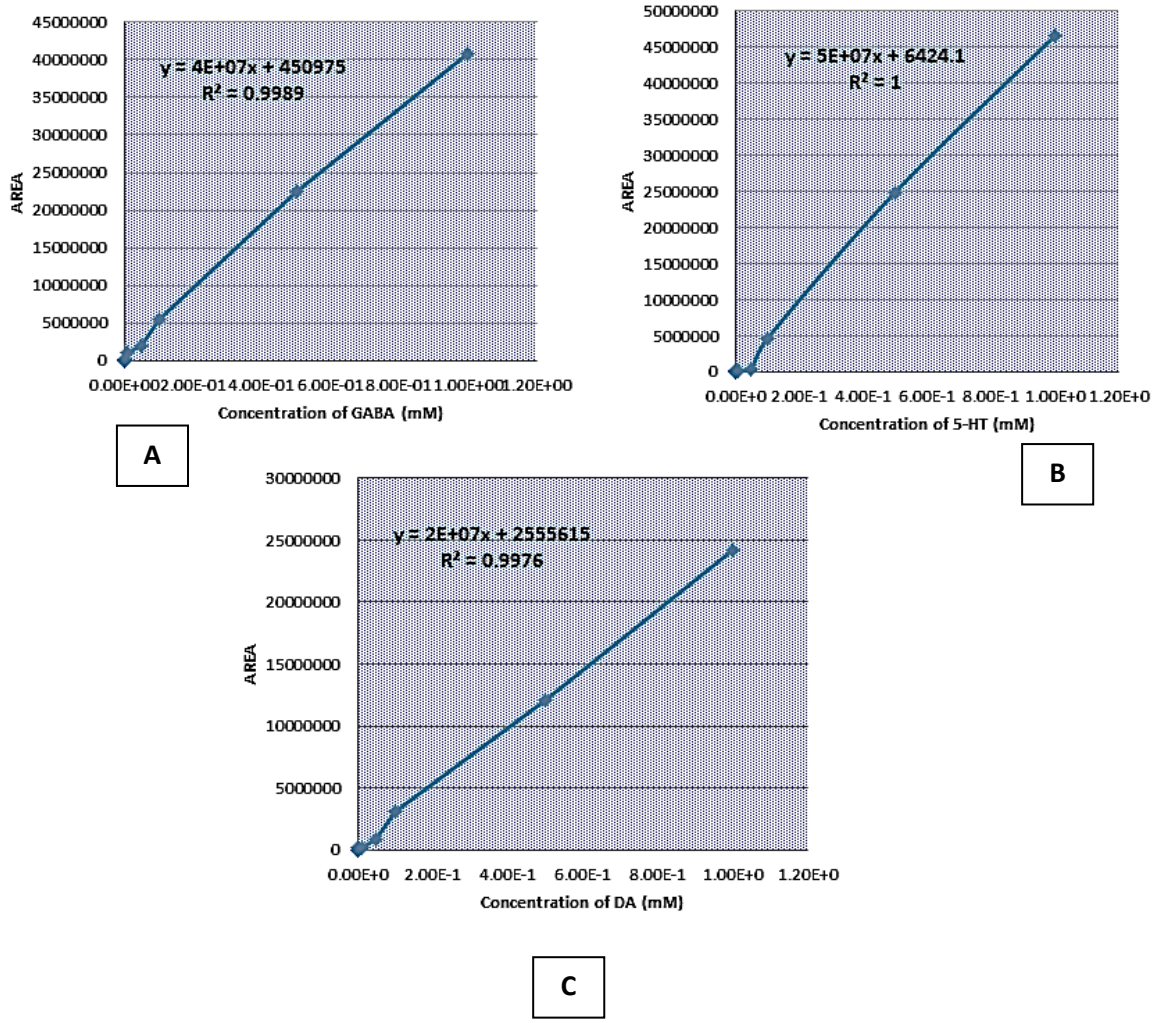


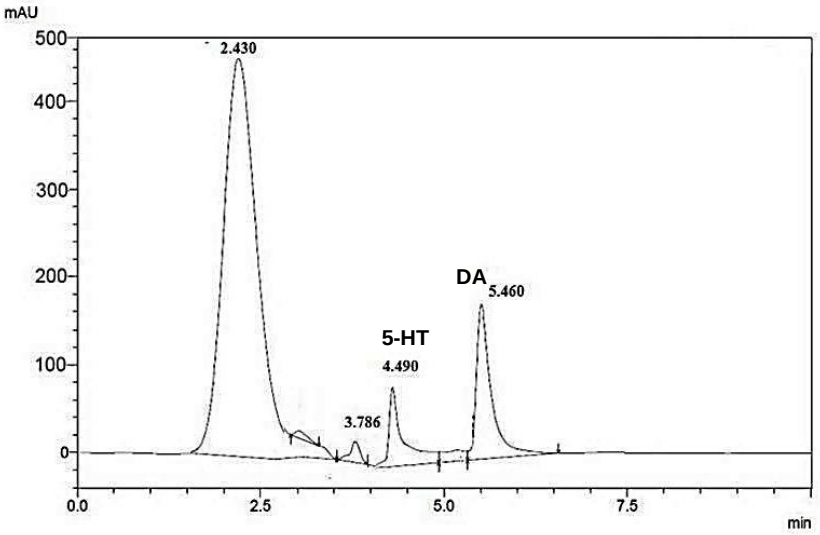
Fig. 6.4. Linear Regression graph of standard neurotransmitters. **A)** GABA; **B)** 5-HT; **C)** DA

PDA analysis of levels of standard neurotransmitter in control cells is shown in Fig. 6.5. A chromatogram of neurotransmitters levels in cells treated with various herbal treatments is shown in Fig. 6.6 & 6.7. The conformation of the presence of the neurotransmitters was done by LC-MS analysis (Fig. 6.8). From Table 6.2 and Fig. 6.9, it is evident that herbal drugs like Brahmi, Brahmi ghrita, Brahmi vati and Saraswata ghrita increase the levels neurotransmitter GABA, DA and 5-HT when compared to control sample and two tailed t-test confirmed the significance of the value (Table 6.2). Brahmi increased all the neurotransmitter levels by 30%. While Brahmi ghrita increased 5-HT by 8%, the DA level by 30%. But, the increase in the levels of GABA was not statistically significant. Saraswata Ghrita increased DA and 5-HT by 20% with a statistically insignificant increase in GABA levels. We noticed a major increase in the levels of neurotransmitters by the addition of Brahmi vati. Brahmi vati increased the levels of GABA, DA and 5-HT by 50% (Table 6.2 & Fig. 6.9). The relative increase among the drugs was also significant by ANOVA at α -0.05 (Table 6.2).

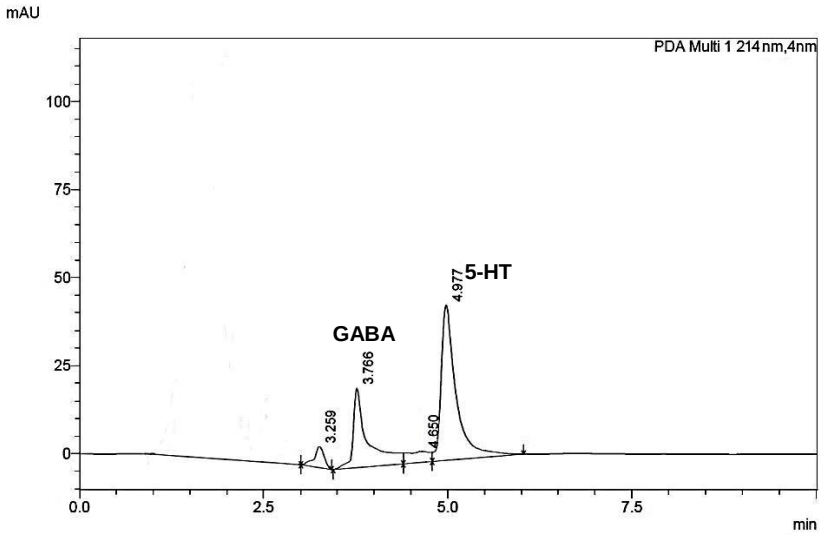
The present study was undertaken to evaluate the effect of Ayurvedic herbal medicines on the neurotransmitter levels in IMR 32, using LC-MS/MS approach. The principal advantages of using LC-MS/MS method include a simple purification procedure and a simple chromatographic condition using the ESI mode. Also, UF-HPLC allows a more rapid analysis and more analyses per day (Guillarme, Ruta, Rudaz, & Veuthey, 2010).

Several LC-MS methods have been reported for the determination of neurotransmitters (Bicker, Fortuna, Alves, & Falcão, 2013; Cifuentes Castro et al., 2014; Foti, Kimura, DeQuattro, & Lee, 1987; Kågedal & Goldstein, 1988; Parrot, Neuzeret, & Denoroy, 2011; Peaston & Weinkove, 2004; Yusof et al., 2000; Zapata, Chefer, Parrot, & Denoroy, 2013), and few for the determination of neurotransmitters from cell cultures (Carrera et al., 2007; Yusof et al., 2000). Our method optimized could use much larger

injection volumes (100 µL), with best separation and in turn lower the detection limits without compromising chromatography sensitivity in a short run time.



A



B

Fig. 6.5. PDA analysis of neurotransmitters in control sample with **A)** 5-HT at 280nm with retention time at 4.49min DA at 280nm with retention time at 5.46min and **B)** GABA at 214nm with retention time at 3.78min,

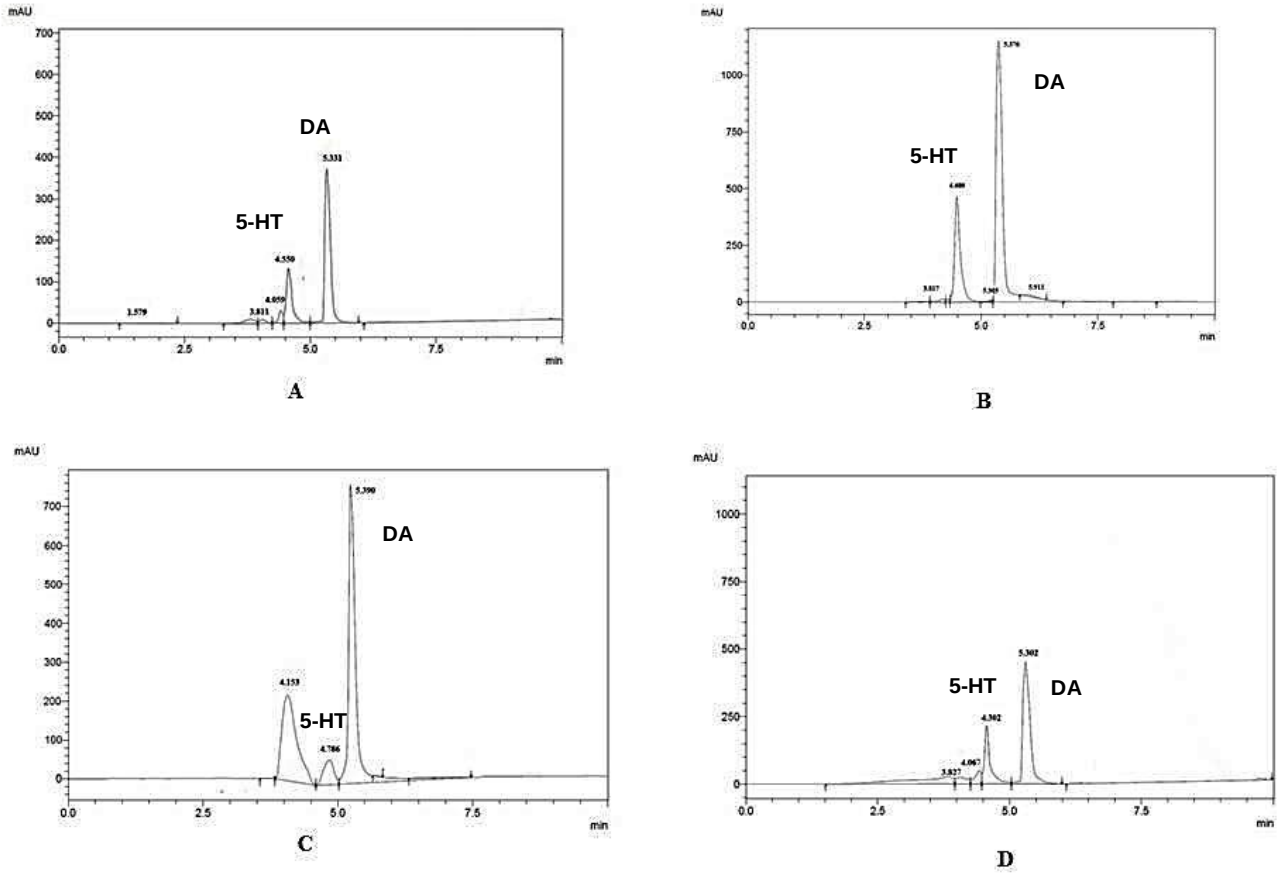


Fig. 6.6. PDA analysis of neurotransmitters at 280nm in different herbal medicines treated cells with their retention time (min). **A)** Bramhi –5-HT – 4.55; DA – 5.33 **B)** Brahmi Vati – 5-HT – 4.5; DA – 5.37 **C)** Brahmi Ghrita – 5-HT – 4.78; DA – 5.39 **D)** Saraswata Ghrita – 5-HT – 4.30; DA – 5.30.

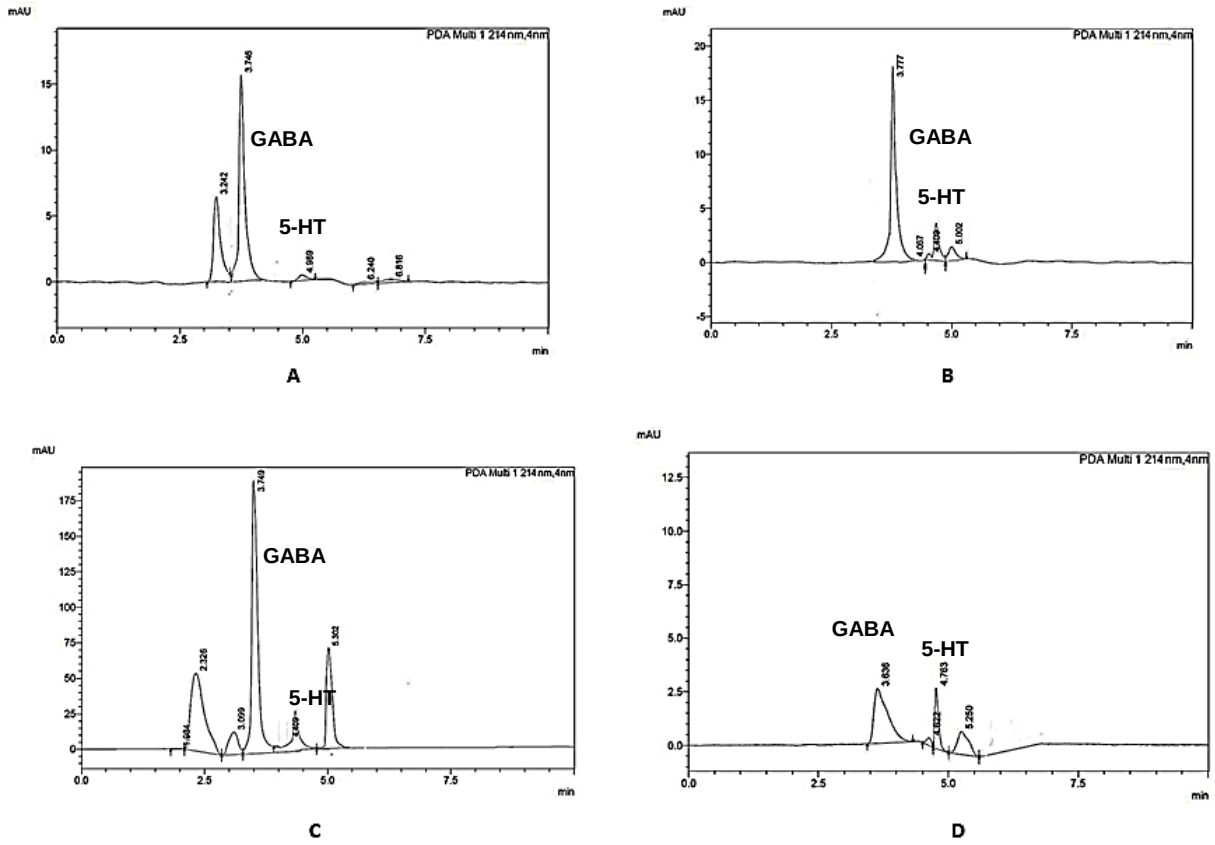


Fig. 6.7 PDA analysis of GABA at 214nm in different herbal medicines treated cells with their retention time (min). **A)** Bramhi – GABA -3.81 **B)** Brahmi Vati – GABA -3.81 **C)** Brahmi Ghrita – GABA – 4.15 **D)** Saraswata Ghrita – GABA -3.82

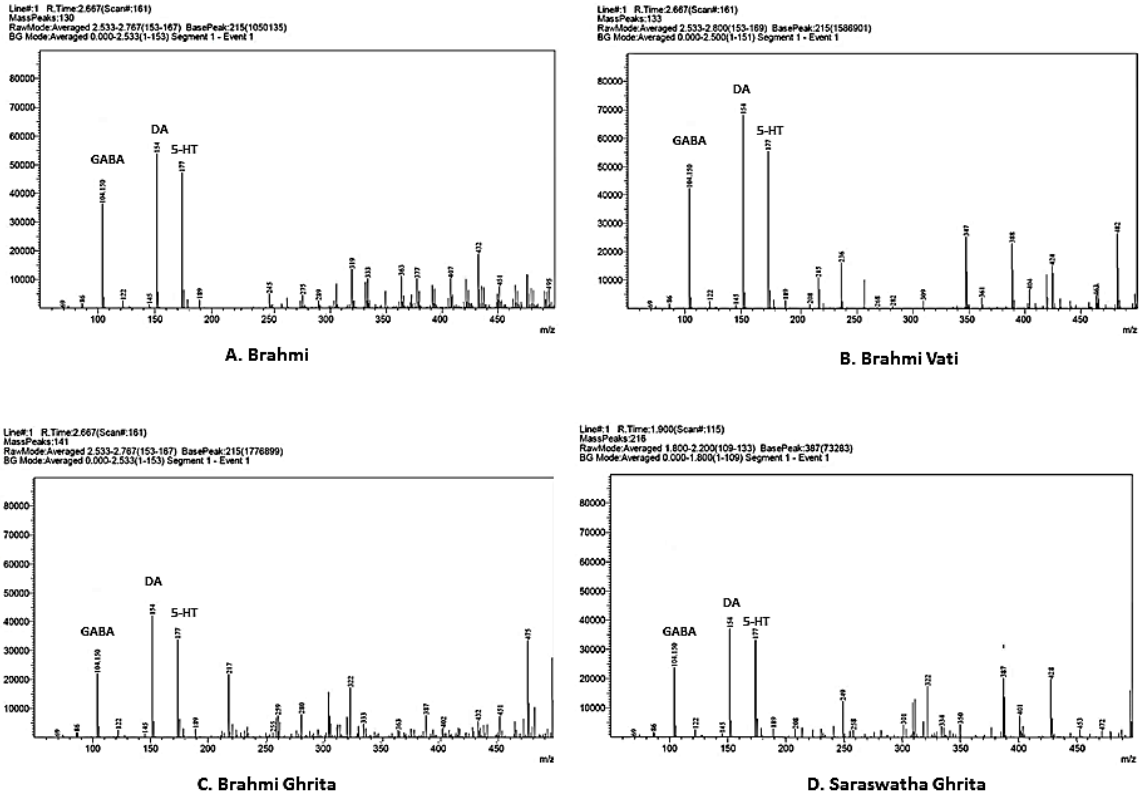


Fig. 6.8. LCMS analysis of neurotransmitters from herbal medicines treated cells. With GABA having mass of 104, 5-HT – 177 and DA – 154.

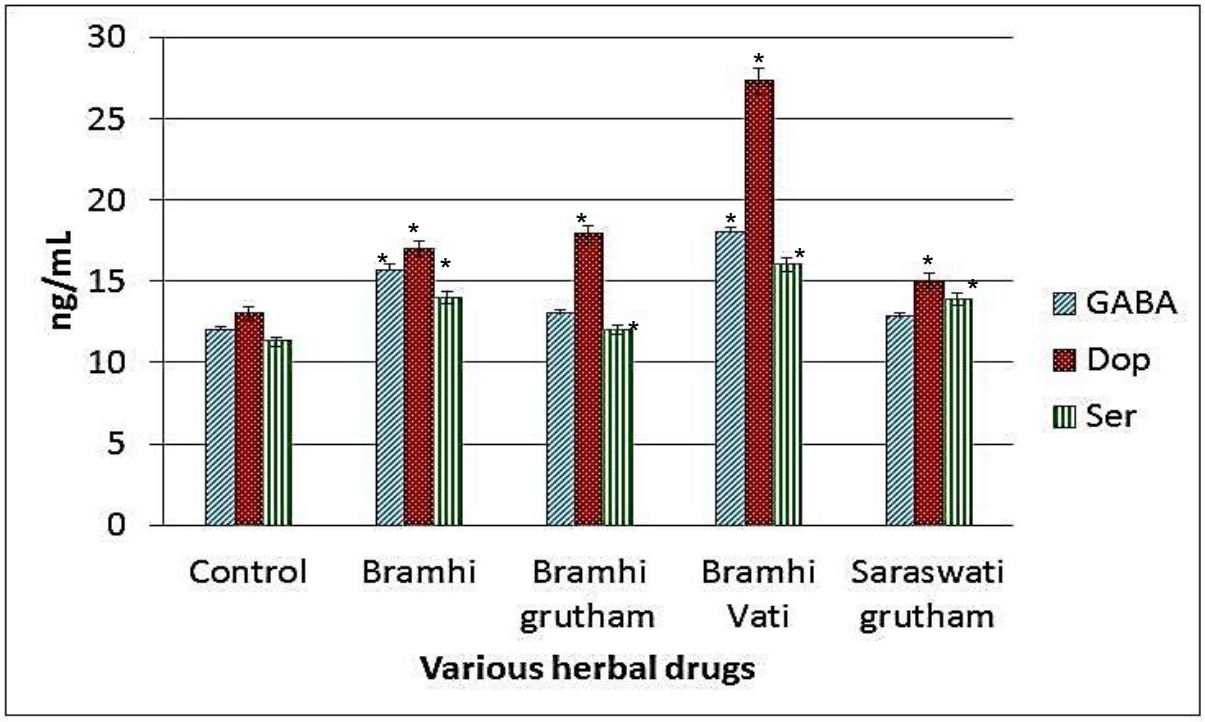


Fig. 6.9 Neurotransmitter levels upon treatment with herbal medicines; * Statistically significant values from T-Test

Table 6.2. Levels of neurotransmitters after treatment with herbal medicines

ng/ml	Control	Bramhi	Bramhi grutham	Bramhi Vati	Saraswati grutham	ANOVA (α - 0.05)
GABA	12.37	15.30 ($\pm 2.4 \times 10^{-3}$)*	12.90 ($\pm 2.3 \times 10^{-1}$)	18.33($\pm 2.6 \times 10^{-4}$)*	12.37 (±1)	6.16×10^{-7}
DOP	13.30	17.33 ($\pm 1.1 \times 10^{-3}$)*	17.37 ($\pm 4.2 \times 10^{-4}$)*	27.80($\pm 3.06 \times 10^{-6}$)*	15.27 ($\pm 2.1 \times 10^{-3}$)*	1.2×10^{-9}
SER	11.07	13.83 ($\pm 8.3 \times 10^{-5}$)*	12.37($\pm 9.4 \times 10^{-3}$)*	16.10 ($\pm 7.54 \times 10^{-6}$)*	13.67 ($\pm 9.7 \times 10^{-3}$)*	5.83×10^{-7}

The parenthesis has 2 tailed unpaired t-test values; * - significant values at P - ≤0.05

Our results suggests that herbal medicines like Brahmi, Brahmi ghrita, Brahmi vati and Saraswata ghrita which are given to children with ASD, increases the neurotransmitter levels in IMR 32 when compared to control cells. Our results also report that Brahmi in poly-herbal form increases the neurotransmitter levels to greater extent than in pure form. Brahmi has been used by Ayurvedic medical practitioners in India for almost 3000 years and is classified as a drug used to improve memory, intellect, anxiety, poor cognition and lack of memory (Russo & Borrelli, 2005). BM is reported to increase the levels of DA and 5-HT in brain (Sheikh et al., 2007) and maintain monoamines homeostasis (Rauf, Subhan, Abbas, Haq, & Ali, 2012)..

Serotonin (5-HT), GABA, Dopamine (DA), Epinephrine (E) and Norepinephrine (NE), Melatonin (M) and cytokines like IL1 and TNF levels play a very important role in regulation behavioral issues like sleep, hyperactivity etc..(Blum et al., 2008; Durant et al., 2010; Ernst et al., 1997; Gold et al., 2014; Hosenbocus & Chahal, 2012; Jouvet, 1972; Kindregan et al., 2015; Krueger et al., 2001; Longordo et al., 2009; Murphy et al., 2004; Yanofski, 2010).

Imbalances in neurotransmitters and cytokines are also implicated in the aetiology of ASD (Ashwood et al., 2011b; Oades et al., 2010; Tsai, 1999), where decreased levels of 5-HT, GABA and DA are reported (Adamsen et al., 2014; Buitelaar & Willemsen-Swinkels, 2000; Gaetz et al., 2014; Harada et al., 2011; Rojas et al., 2014; Veenstra-Vanderweele et al., 2009).

Decreased levels of neurotransmitters are also reported to be involved in the aetiology of behavioral abnormalities as observed in comorbid conditions of ASD. With our study reporting that Brahmi helps in increasing the levels of 5-HT, GABA and DA, the prescription of this herbal drug might in turn help in alleviating the behavioral problems in children with ASD stands validated.

6.4. Conclusion

All herbal drugs used in this study increased the neurotransmitters DA, 5-HT and GABA levels. Brahmi was the major component of all the drugs analysed and it was found to be more effective in poly-herbal form than in pure form. Our study is limited to the detection of only three neurotransmitters and not their metabolites. Our research suggests genetic analysis of the rate limiting enzymes in the neurotransmitter production to further substantiate our results.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The first chapter gives a comprehensive overview of Autism Spectrum Disorder, its core features, aetiology, prevalence, various risk factors and comorbid conditions of ASD in Indian scenario. The aetiology of ASD remains unclear and the reported brain abnormalities among children with ASD indicate a probable link with disturbances in the in utero period. Hence, it becomes logical to explore prenatal, perinatal and neonatal risk factors of ASD. Apart from typical ASD symptoms, there exist comorbid conditions among children with ASD. These behavioral abnormalities are one of the major challenges faced by the parents and caregivers. Thus, it is necessary to explore this area. Due to the side effects of allopathic medicines, parents are opting for alternate medicine like Ayurveda to treat these behavioral issues. Scientific reports suggests role of neurotransmitters in these behavioral issues and ASD. Despite of extensive work, there are limited published reports regarding impact of herbal medicines at cellular and molecular levels. Also, there is lack of literature regarding the various herbal medicines prescribed to children with ASD. Therefore, evaluation of the effect of herbal medicines on neurotransmitter level is a prime area of research to focus on.

The second chapter of this thesis deals with validation of the questionnaire developed. Content validity was tested by percentage agreement and content validity index. The reliability of the method was tested by internal consistency and test-retest procedure. The data was collected from various autism centers and schools across 9 cities using this validates questionnaire. The questionnaire was found to be reliable and suitable for collecting data on risk factors of ASD.

The third chapter of this thesis explores parental risk factors of ASD. With genetics and hormonal disturbances playing a major role in the aetiology of ASD, there is a need to look into parental history. Our factors under study like Advanced maternal age, consanguinity among parents, polycystic ovarian syndrome and maternal hormonal interventions were very

significant risk factors for ASD, while advanced parental age and maternal thyroid hormone imbalance did not contribute towards ASD risk.

The fourth chapter describes about prenatal, perinatal and neonatal risk factors of ASD. With existence of multiple causal factors and the reported brain abnormalities among children with ASD suggesting a probable link with disturbances in the *in utero* period, it becomes necessary to explore prenatal, perinatal and neonatal risk factors of ASD. Among the prenatal factors considered, fetal distress and gestational respiratory infections were found to be associated with ASD, while among perinatal and neonatal risk factors labor complications, pre-term birth, neonatal jaundice, delayed birth cry and birth asphyxia were found to be associated with ASD risk. Our study advocates additional focused investigations on physiological and genetic changes contributed by these risk factor inducing environments.

The chapter five is about parental perspectives of comorbid challenges in ASD. Due to lack of awareness about the comorbid conditions of ASD, and the existence of several cultural beliefs and myths in India, it becomes important to understand and educate parents and professionals about these conditions. We evaluated factors which have a likely link to behavioral issues like Sleep disturbances, Constipation, Food allergies, Skin allergies, Attention deficit hyperactivity. Apart from existing independently, these factors co-existed with each other. Educating parents and professionals about these conditions would in turn help in designing effective and economic intervention services.

The chapter six deals with evaluation of the molecular effects of ayurvedic medicines on neurotransmitter production in IMR 32 cell line. ASD is associated with comorbid conditions which induce behavioral issues. Behavioral issues are reported to involve alterations in neurotransmitter levels. Also decreased dopamine, GABA and serotonin levels are found in ASD cases. Perceptions of safe usage of alternate medicine like Ayurveda encourage the wider usage of alternate medicine for the treatment of behavioral issues. With

non-availability of data to prove exact action of herbal medicines prescribed to alleviate behavioral abnormalities, our study evaluated the effect of herbal medicines on neurotransmitter production. Brahmi is reported to be one of the important herbal medicines for treating CNS disorders. It is also reported improve behavioral alterations and oxidative markers in sodium valproate induced autism in rats. Thus, we analysed the effect of Brahmi, Brahmi vati, Brahmi ghrita and Saraswata ghrita (all of them have brahmi as major component) on neurotransmitter production in IMR 32 cell line. All herbal medicines increased neurotransmitter levels in IMR 32 with BV having maximum effect indicating that the prescription of this herbal drug might in turn help in alleviating the behavioral problems in children with ASD.

Limitations

- Our study on risk factors is limited by certain constraints like vastness of the region with ethnically different population, no published report on incidence and prevalence of ASD, the non-availability of structured and reliable record keeping on maternal and fetal conditions in the country, dependence on maternal memory for data acquisition.
- Statistically, significant risk association was found for PCOS, hormonal interventions in mother and the aetiology of ASD in child, but more extensive studies on quantitative assessment in terms of dosage, treatment regime and its outcome needs to be evaluated.
- The results on increased neurotransmitter levels should be further substantiated genetic studies and *in vivo* studies.

Specific Contribution

- Our study is the first report on risk factors of ASD. Out of all the factors analyzed, 12 factors, Advanced maternal age, consanguinity, PCOS, maternal hormonal interventions, fetal distress and gestational respiratory, labor complications, pre-term birth, neonatal jaundice, delayed birth cry and birth asphyxia were the significant ones.
- This is the first study in India, documenting the existence of comorbid conditions among children with ASD.
- Our study is first of its kind in reporting the increase in neurotransmitter levels by four herbal medicines Brahmi, Brahmi ghrita, Brahmi vati and Saraswata ghrits.

Scope for future work

ASD is multifactorial neurodevelopmental disorder and its aetiology is not known. A lot of research is going on to understand ASD.

Future work in risk factors

We analysed the presence of risk factors but their genetic and environmental components are still under research.

1. Genetic association of consanguinity for the risk of ASD. Consanguinity is known for the risk of autosomal recessive disorder, but this area is not explored regarding ASD risk.
2. Genetic association of conditions like gestational diabetes and hypertension in mother to the risk of ASD is yet to be analyzed.
3. Very primitive data is available of the effect of hormonal conditions like PCOS on the risk of ASD. This, this is a very potential area. Also limited information is available regarding biomarkers for ASD.

Future work in comorbid conditions

We analysed only those conditions which had behavioural impact, but other conditions anxiety, depression, bipolar disorders, intellectual disability, bowel disease, Tourette syndrome etc. are yet to be studied in Indian population.

Future work in Herbal medicine and ASD

Genetic analysis of the rate limiting enzymes in the neurotransmitter production upon treatment with herbal medicines is yet to be evaluated.

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APPENDIX I

APPENDIX II

APPENDIX III

APPENDIX IV

APPENDIX V

LIST OF PUBLICATIONS

Publications

1. **Madhu Poornima Mamidala**, Mahesh Kumar Kalikiri, Praveen Kumar P. T. V., N Rajesh, Om Sai Ramesh Vallamkonda, Vidya Rajesh, "Consanguinity in India and its association with Autism Spectrum Disorder", **Autism Research**, 8, (2015), 224 – 228.
2. **Madhu Poornima Mamidala**, Anupama Polinedi, Praveen Kumar P.T. V, Rajesh N., Om Sai Ramesh Vallamkonda, Vrajesh Udani, Nidhi Singhal, Vidya Rajesh, " Maternal hormonal interventions as a risk factor for Autism Spectrum Disorder: an epidemiological assessment from India." **Journal of Biosciences**, 38 (5), (2013), 887 - 892.
3. **Madhu Poornima Mamidala**, Anupama Polinedi, Praveen Kumar. P. T. V, Rajesh N., Om Sai Ramesh Vallamkonda, Vrajesh Udani, Nidhi Singhal, Vidya Rajesh, "Prenatal, perinatal, and neonatal risk factors of Autism Spectrum Disorder: A comprehensive epidemiological assessment from India", **Research in Developmental Disabilities**, 34 (9), (2013), 3004-3013.
4. **Madhu Poornima Mamidala**, Mahesh Kalikiri, Praveen K. PTV, Vidya Rajesh, "Parental perspectives of comorbid challenges in Autism Spectrum Disorder: Findings from India". (Under review)
5. **Madhu Poornima Mamidala**, N Rajesh, Vidya Rajesh, "Evaluation of the molecular effects of Ayurvedic medicines on neurotransmitter production in IMR 32 cell line". (Under Review).
6. Ramani Lakshmi Aluru, **Madhu Poornima Mamidala**, N Rajesh, Vidya Rajesh, "Detection and analysis of neurotransmitters and their metabolites using UFLC-ESI/MS with UV detection". (Under review).
7. Mahesh Kumar Kalikiri, **Madhu Poornima Mamidala**, Ananth N Rao, Vidya Rajesh, "Novel Polymorphisms in the Ligand Binding Domain and DNA Binding Domain of Thyroid hormone receptors in Patients with Autism Spectrum Disorder". (Under Review).

Seminars

1. **Madhu Poornima Mamidala**, Mahesh Kumar Reddy, Vidya Rajesh, "An epidemiological study on maternal polycystic ovarian syndrome as risk factor for Autism Spectral Disorder," International symposium - Evidence in Global Disability and Health, Hyderabad Central University, 22-23rd February, 2014.
2. **Madhu Poornima Mamidala**, Mahesh Kumar Reddy K., Praveen Kumar PTV, N. Rajesh, Vidya Rajesh, "Thyroid malfunction as a risk factor for Autism Spectrum Disorder", International Conference on Cerebral Palsy and developmental medicine, Lucknow, March 6 -10, 2013.
3. **Madhu Poornima Mamidala**, C.N. Rahul, N. Rajesh, Vidya Rajesh, "Assessment of Assisted Reproductive Technology and its associated risk with Autism Spectral Disorder in Indian population", European Human Genetics Conference, 28th – 31st May, 2011, Amsterdam, The Netherlands. Abstracts published in European Journal of Human Genetics, Volume 19, Supplement 2, May 2011.

BIOGRAPHY

Biography of Prof. Vidya Rajesh

Prof. Vidya Rajesh is Associate Professor in the Department of Biological Sciences. She is also Associate Dean of Academic Research Division of BITS, Pilani – Hyderabad Campus. Prof. Vidya Rajesh completed her M. Sc. in Microbiology from Nagpur University in the year 1995 and did her M. E. in Biotechnology (2000) and Ph. D from BITS, Pilani – Pilani campus in the year 2007. The topic of her doctoral thesis was *"Studies on Sequence Diversity and Characterization of the Apical Membrane Antigen of Plasmodium in Indian Isolates".* She has been actively involved into academics and has gained 13 yrs of teaching, research and administrative experience during her tenure in various capacities at BITS. Her current research interest is in the area of molecular genetics of human diseases. Her initial interest was only in the area of genetics of infectious disease like Malaria from Indian isolates and later diversified to Autism and Environmental bioremediation. She has nearly 25 research publications and equal number of conference presentations. She has completed three research projects as principal investigator and has an ongoing DST project as co-investigator. Her UGC project provided the funding for this work on risk factors of Autism. She has extended her work on this area and is focusing on projects like thyroid receptors mutations in autism and analysis of urinary biomarkers in autistic patients. She currently has three Ph.D students working under her supervision. Prof. Vidya Rajesh is also part of other institutional projects like DST – FIST and DST – TBI at BITS, Pilani – Hyderabad campus.

Biography of Ms. M. Madhu Poornima

Mrs. M. Madhu Poornima is a Ph. D student in the Department of Biological Sciences. She pursued her M. Sc. in Biochemistry from Osmania University in the year 2008 and stood first in her university. Madhu joined BITS, Pilani Hyderabad campus in a UGC project at Department of Biological Sciences and continued her work as INSPIRE fellow (JRF and SRF) for her doctoral degree. She is well versed with statistics, animal tissue culture and liquid chromatography mass spectroscopy. Her experience in various techniques is evident from good number of publications and seminars attended (national and International). Currently her research interests focus on epidemiology of diseases and mass spectroscopy.