EFFECT OF ADJUNCTIVE VITAMIN D SUPPLEMENTATION AS MEASURED BY SERUM VITAMIN D AND NGF LEVELS IN AUTISM SPECTRUM DISORDER: AN OPEN-LABEL STUDY TRIAL

By

Dr. Kanchan Garg

under the guidance of

Dr. Varun S. Mehta,

Professor of Psychiatry,
Central Institute of Psychiatry, Ranchi

Joint guidance of

Dr. K. K. Kshitiz,

Professor of Biochemistry,
Central Institute of Psychiatry, Ranchi

CENTRAL INSTITUTE OF PSYCHIATRY
RANCHI-834006, JHARKHAND (INDIA)

THESIS

SUBMITTED TO RANCHI UNIVERSITY, RANCHI
IN PARTIAL FULFILMENT FOR THE DEGREE OF
DOCTOR OF MEDICINE
(PSYCHIATRY)
2022-2025

GOVERNMENT OF INDIA

CENTRAL INSTITUTE OF PSYCHIATRY

RANCHI-834006, JHARKHAND, INDIA

Declaration

I hereby declare that the present study titled **"EFFECT OF ADJUNCTIVE VITAMIN D SUPPLEMENTATION AS MEASURED BY SERUM VITAMIN D AND NGF LEVELS IN AUTISM SPECTRUM DISORDER: AN OPEN-LABEL STUDY TRIAL"** has been conducted by me at the Central Institute of Psychiatry, Ranchi under the guidance of Dr. Varun S. Mehta, Professor of Psychiatry, Central Institute of Psychiatry and Dr. K.K. Kshitiz, MD, Professor of Biochemistry, Central Institute of Psychiatry.

I further declare that this is an original study and no part of it has been published or submitted to any university previously.

Place: Ranchi, Jharkhand ***Dr. Kanchan Garg***

Date:

GOVERNMENTOFINDIA

CENTRAL INSTITUTE OF PSYCHIATRY

RANCHI-834006, JHARKHAND, INDIA

Certificate

This is to certify that **Dr. Kanchan Garg** is a **Bonafide student** at **Central Institute of Psychiatry**, Ranchi, pursuing the course of Doctor of Medicine (M.D.) of Ranchi University for the session 2022-2025. She has carried out this study titled **"EFFECT OF ADJUNCTIVE VITAMIN D SUPPLEMENTATION AS MEASURED BY SERUM VITAMIN D AND NGF LEVELS IN AUTISM SPECTRUM DISORDER: AN OPEN-LABEL STUDY TRIAL"** at the institute under our supervision. This thesis is hereby approved for submission to Ranchi University as partial fulfillment of the requirements of Doctor of Medicine (M.D).

Dr Varun S. Mehta,
Professor of Psychiatry,
Central Institute of Psychiatry, Ranchi

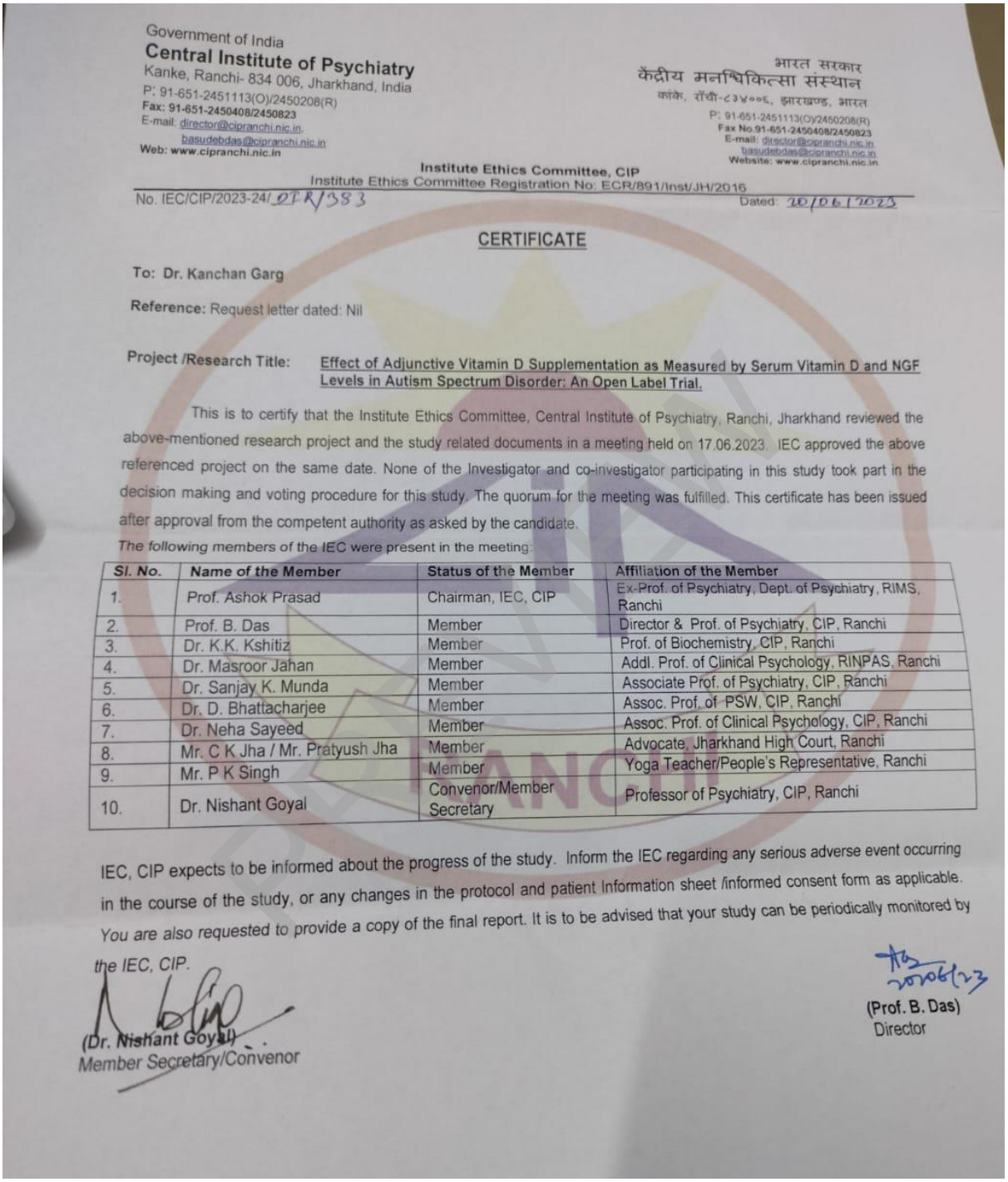
Dr. K. K. Kshitiz, M.D
Professor of Biochemistry,
Central Institute of Psychiatry, Ranchi

Place: Ranchi, Jharkhand

Date:

CERTIFICATE OF ETHICS COMMITTEE APPROVAL

ETHICS COMMITTEE APPROVAL



Government of India
Central Institute of Psychiatry
Kanke, Ranchi- 834 006, Jharkhand, India
P: 91-651-2451113(O)/2450208(R)
Fax: 91-651-2450408/2450823
E-mail: director@cipranchi.nic.in,
basudebdas@cipranchi.nic.in
Web: www.cipranchi.nic.in

भारत सरकार
केंद्रीय मनश्चिकित्सा संस्थान
कांके, राँची-८३४००६, झारखण्ड, भारत
P: 91-651-2451113(O)/2450208(R)
Fax No.91-651-2450408/2450823
E-mail: director@cipranchi.nic.in
basudebdas@cipranchi.nic.in
Website: www.cipranchi.nic.in

Institute Ethics Committee, CIP
Institute Ethics Committee Registration No: ECR/891/Inst/JH/2016

No. IEC/CIP/2023-24/ DR/383 Dated: 20/06/2023

CERTIFICATE

To: Dr. Kanchan Garg

Reference: Request letter dated: Nil

Project /Research Title: Effect of Adjunctive Vitamin D Supplementation as Measured by Serum Vitamin D and NGF Levels in Autism Spectrum Disorder: An Open Label Trial.

This is to certify that the Institute Ethics Committee, Central Institute of Psychiatry, Ranchi, Jharkhand reviewed the above-mentioned research project and the study related documents in a meeting held on 17.06.2023. IEC approved the above referenced project on the same date. None of the Investigator and co-investigator participating in this study took part in the decision making and voting procedure for this study. The quorum for the meeting was fulfilled. This certificate has been issued after approval from the competent authority as asked by the candidate.

The following members of the IEC were present in the meeting:

Sl. No.	Name of the Member	Status of the Member	Affiliation of the Member
1.	Prof. Ashok Prasad	Chairman, IEC, CIP	Ex-Prof. of Psychiatry, Dept. of Psychiatry, RIMS, Ranchi
2.	Prof. B. Das	Member	Director & Prof. of Psychiatry, CIP, Ranchi
3.	Dr. K.K. Kshitiz	Member	Prof. of Biochemistry, CIP, Ranchi
4.	Dr. Masroor Jahan	Member	Addl. Prof. of Clinical Psychology, RINPAS, Ranchi
5.	Dr. Sanjay K. Munda	Member	Associate Prof. of Psychiatry, CIP, Ranchi
6.	Dr. D. Bhattacharjee	Member	Assoc. Prof. of PSW, CIP, Ranchi
7.	Dr. Neha Sayeed	Member	Assoc. Prof. of Clinical Psychology, CIP, Ranchi
8.	Mr. C K Jha / Mr. Pratyush Jha	Member	Advocate, Jharkhand High Court, Ranchi
9.	Mr. P K Singh	Member	Yoga Teacher/People's Representative, Ranchi
10.	Dr. Nishant Goyal	Convenor/Member Secretary	Professor of Psychiatry, CIP, Ranchi

IEC, CIP expects to be informed about the progress of the study. Inform the IEC regarding any serious adverse event occurring in the course of the study, or any changes in the protocol and patient Information sheet /informed consent form as applicable. *You are also requested to provide a copy of the final report. It is to be advised that your study can be periodically monitored by the IEC, CIP.*

(Dr. Nishant Goyal)
Member Secretary/Convenor

20/06/23
(Prof. B. Das)
Director

ACKNOWLEDGEMENT

First and foremost, I bow in gratitude to the Almighty, whose constant blessings and guidance have been my strength throughout this journey.

I am deeply indebted to my guide, Prof (Dr.) Varun S. Mehta, for his invaluable mentorship, his unwavering support, constructive criticism, and insightful suggestions shaped the foundation of this research. I am equally thankful to my co-guide, Prof (Dr.) K. K. Kshitiz, whose thoughtful feedback and detailed inputs greatly enhanced the quality of this work.

I wish to acknowledge the encouragement of my parents, whose love and faith have been my anchor. I am also grateful to my siblings, Dr. Punam and Riddhima, whose support and motivation constantly inspired me to persevere.

My heartfelt thanks go to my colleagues, seniors, and juniors for their guidance. I am particularly thankful to Mr. Nagendra, Mr. Virendra, Mr. Rohit, and Mr. Umesh, along with the hospital staff, for their cooperation, which was indispensable to the smooth conduct of this study.

Above all, I owe special gratitude to the patients who participated in this research. Their willingness to contribute despite personal challenges has been deeply humbling.

Lastly, I extend warm thanks to my friends, extended family, and well-wishers, whose encouragement and belief in me provided constant motivation. This achievement is shared with all who stood by me on this path.

Dr. Kanchan Garg

CONTENTS

Chapter 1: Introduction

INTRODUCTION

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders characterized by impaired social interaction and communication, repetitive and stereotyped behaviors, limited interests, and abnormalities in sensory processing, generally occurring in early childhood (lord et al.,2018). It has been hypothesized that ASD is a combination of both organ specific physiologic and systematic abnormalities such as de novo gene mutations, oxidative stress, impaired detoxification system, inflammation, immune dysregulation, abnormal neurotrophic factor and neurotransmitter levels, and seizures, at least in a subset of individuals with ASD (Da Rossignol etal.,2014).

In recent years, vitamin D has been studied as one of the important biological factors related to ASD. Apart from its role in bone and calcium metabolism, vitamin D also acts like a hormone in the brain and influences growth and development of nerve cells (Eyles et al., 2013). Children with ASD are more likely to have low vitamin D because of less outdoor activity and sun exposure, selective food habits, stomach or gut-related problems that affect absorption, and genetic variations in vitamin D metabolism. A study by Schmidt et al. (2015) showed that certain gene variants linked to vitamin D metabolism were associated with higher risk of ASD.

Low vitamin D can interfere with several important processes in the brain. It has been linked to reduced serotonin synthesis, a neurotransmitter that plays a key role in mood regulation, impulse control, and social behavior (Patrick et al., 2015). Deficiency is also associated with higher levels of oxidative stress and inflammation, which may further disrupt neuronal function (Ansari et al., 2020). Moreover, vitamin D is required for the production of neurotrophins such as nerve growth factor (NGF), which support neuronal growth, survival, and connectivity (Gezen et al., 2014; Huang et al., 2001).

Restoring vitamin D levels through supplementation may help correct these disturbances. Adequate vitamin D has the potential to normalize serotonin activity, reduce oxidative stress, regulate immune responses, and enhance NGF expression. Through these mechanisms, supplementation could contribute to improvement in behavioral symptoms of ASD, including irritability, hyperactivity, and social difficulties. Clinical studies evaluating vitamin D supplementation have reported encouraging findings, although results remain mixed, with some trials showing benefit and others failing to confirm significant effects (Kittana et al., 2021). In this context, the present study was undertaken to examine whether vitamin D

supplementation can improve behavioral outcomes and influence biological markers such as NGF in children with ASD.

Need for study

Vitamin D deficiency has been proposed as a risk factor for several neurodevelopmental and psychiatric disorders, including ASD (Cannell, 2008; Eyles et al., 2013; Saad et al., 2016). However, a few studies have not found this association (Molloy et al., 2010; Hashemzadeh et al., 2015). Till now, around twelve clinical trials have examined the role of vitamin D supplementation in children with ASD (Kittana et al., 2021). Although many of these reported positive effects, their results remain inconsistent because of methodological shortcomings such as small sample sizes, case-series designs (Saad et al., 2016), non-adherence to standard dosing guidelines (Feng et al., 2017; Bleizgys, 2021), use of concurrent supplementation like omega-3 fatty acids, and lack of consistent correlation between serum vitamin D levels and ASD severity (Mazahery et al., 2019; Javadfar et al., 2020).

Only one study so far has explored the effect of vitamin D supplementation on NGF levels in ASD and reported a significant increase (Ucuz et al., 2014). This is important because NGF is a key neurotrophins involved in brain growth and connectivity, and its modulation by vitamin D may provide a biological explanation for clinical improvement.

Despite this, there is a lack of evidence from India, where both ASD prevalence and vitamin D deficiency are common. This highlights the need for further research in this area. The present study was therefore undertaken to evaluate the effects of vitamin D supplementation on behavioral outcomes and serum biomarkers, specifically serum 25-hydroxycholecalciferol and NGF.

Chapter 2: Review of literature

2.1 Autism Spectrum Disorder (ASD)

- 2.1.1 Introduction
- 2.1.2 Sociodemographic Characteristics of ASD

2.2 Vitamin D and Autism Spectrum Disorder

- 2.2.1 Vitamin D: Introduction
- 2.2.2 Role of Vitamin D in ASD
- 2.2.3 Serum Vitamin D Levels in Children with ASD
- 2.2.4 Vitamin D Supplementation as an Adjunctive Intervention in ASD: Evidence from Interventional Trials

2.3 Nerve Growth Factor (NGF) and autism spectrum disorder

- 2.3.1 NGF: Introduction
- 2.3.2 Role of NGF in ASD
- 2.3.3 Vitamin D Regulation of NGF in the Context of Neurodevelopment and ASD

2.1 autism spectrum disorder (ASD)

- **2.1.1 Introduction**

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in the ability to initiate and sustain reciprocal social interaction and communication, along with restricted, repetitive, and inflexible patterns of behavior, interests, or activities (World Health Organization, 2019). These symptoms are present from early developmental periods and cause significant impairment in personal, family, social, educational, or occupational functioning.

ASD can typically be diagnosed as early as 12 to 24 months of age when distinguishing features begin to separate from normative developmental trajectories. In majority of cases, expression of full symptom usually becomes evident later in childhood when social demands increase or compensatory strategies fail. The clinical course and manifestation of ASD vary widely based on developmental stage, symptom severity, cognitive level, and environmental context.

Apart from the core diagnostic criteria, individuals with ASD frequently present with wide range of comorbidities and associated features. These may include intellectual disability, anxiety disorders, attention-deficit/hyperactivity disorder (ADHD), depressive symptoms, epilepsy, sensory processing abnormalities, gastrointestinal disturbances, aggressive behavior, and sleep dysregulation. Immune dysregulation and altered metabolic pathways have also been implicated in subsets of individuals with ASD, further underscoring the condition's biological complexity (Leyfer et al., 2006; Simonoff et al., 2008).

This phenotypic and etiological variability highlights the importance of adopting a personalized, multidisciplinary approach to assessment and intervention in ASD. Understanding the neurodevelopmental basis and clinical breadth of ASD provides a crucial foundation for exploring modifiable risk factors, including nutritional and neuroimmune contributors such as vitamin D and neurotrophic signaling.

2.1.2 Sociodemographic Characteristics of Autism Spectrum Disorder

The global prevalence of Autism Spectrum Disorder (ASD) has risen sharply in recent decades, largely due to increased awareness, broader diagnostic criteria, and improved screening practices. According to the U.S. Centers for Disease Control and Prevention (CDC, 2023), ASD affects approximately 1 in 36 children (2.8%) in the United States, reflecting a substantial increase from earlier decades. This rise is considered to be the result of enhanced surveillance rather than a true increase in incidence. Over the past few decades, the reported prevalence of Autism Spectrum Disorder (ASD) has risen sharply worldwide. While this increase partly reflects better awareness, broader diagnostic criteria, and systematic screening, it also highlights genuine differences in detection between regions. In the United States about 1 in 36 children (2.8%) meet diagnostic criteria (Centers for Disease Control and Prevention [CDC], 2023), and similar upward trends are seen across other high-income countries, where prevalence ranges from 0.7% to 1.5% in Europe and from 1% to 2.6% in East Asia (Elsabbagh et al., 2012; Kim et al., 2011; Sun et al., 2019). In many low- and middle-income countries (LMICs), however, rates appear lower, not necessarily because ASD is less common, but because access to screening is limited, trained specialists are scarce, and sociocultural stigma remains a barrier to seeking diagnosis (Durkin et al., 2015). Even so, the overall burden is considerable, with the Global Burden of Disease 2019 Autism Collaborators (2022) estimating that more than 62 million people worldwide are currently living with ASD or related neurodevelopmental conditions.

India reflects this global pattern of increasing prevalence but with substantial regional variation. Nationally, a meta-analysis in *Indian Pediatrics* estimated a prevalence of 1.12% among children aged 2–9 years (Raina et al., 2017). Local studies add important nuance: in Chandigarh, 0.225% of children were identified with ASD (Arora et al., 2018); pooled estimates suggest slightly higher prevalence in rural areas (0.11%) compared with urban settings (0.09%); and in Himachal Pradesh, rural prevalence (0.24%) is more than double the urban figure (0.09%) (Raina et al., 2021). These patterns suggest that access to diagnostic services, health infrastructure, and public awareness vary considerably between regions, influencing who is identified and when.

Beyond these geographic differences, consistent sex-based disparities are observed in both global and Indian data. Males are diagnosed with ASD about three to four times more often than females (Loomes et al., 2017; Chattopadhyay, 2024). This gap is not fully explained by

biology; in many cases, it reflects under-recognition in females, whose autistic traits may be subtler or masked by adaptive social behaviors. Such masking can delay diagnosis, contributing to another common pattern: even when early signs appear between 6 and 18 months of age, many children—particularly in LMICs—are diagnosed years later. In India, the mean age at diagnosis exceeds four years (Chillage et al., 2025), with key contributors including limited public awareness, the absence of routine developmental screening, and persistent shortages of trained professionals, especially in rural and tribal areas (Kumar et al., 2016; Emerson et al., 2019).

Socioeconomic conditions further interact with these delays. Families living in urban areas or with higher incomes are more likely to access diagnostic assessments early, benefiting from better healthcare and educational resources. By contrast, children from economically disadvantaged or marginalized communities face the same delays described earlier, compounded by reduced availability of intervention services (Gupta et al., 2021).

Adding to these sociodemographic influences are maternal health factors during pregnancy, which have been linked to ASD risk in multiple studies. Advanced maternal age (over 35 years) modestly increases the likelihood of ASD, possibly due to accumulated genetic mutations or obstetric complications (Sandin et al., 2012). Other risk factors include prenatal exposure to medications such as valproic acid, maternal infections during the first or second trimester (Atladottir et al., 2010; Christensen et al., 2013), metabolic conditions like obesity and diabetes, extreme prematurity, and complications such as preeclampsia (Kalkbrenner et al., 2014). While biologically plausible, most of these associations have modest effect sizes, and causality remains under investigation.

Taken together, these patterns show that ASD detection and outcomes are shaped by a complex interplay of geography, gender, age at diagnosis, socioeconomic status, and maternal health. In high-income countries, earlier identification has improved access to intervention, but in LMICs such as India, systemic barriers still delay diagnosis. Addressing these barriers will require nationally coordinated screening programmes, expanded training for primary healthcare workers, and culturally relevant public education. At the same time, there is an urgent need for large-scale, longitudinal research in India to better define local risk profiles and inform evidence-based early intervention.

2.2. Vitamin D and Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is increasingly understood as a multifactorial neurodevelopmental condition which results from dynamic interactions between genetic vulnerability and environmental influences, including immunological, nutritional, and epigenetic factors (Bölte et al., 2019; Lyall et al., 2017). Among these, vitamin D deficiency, particularly during critical periods of prenatal and early postnatal brain development has gained attention as a potentially modifiable risk factor (Cannell et al., 2008; Zerbo et al., 2015).

Various molecular, epidemiological, and interventional studies suggest that inadequate vitamin D levels may negatively influence neurodevelopment in the general population. Proposed mechanisms include alterations in neuroimmune modulation, disruptions in synaptic regulation, and changes in gene expression control (Eyles et al., 2005; McGrath et al., 2001; Harms et al., 2011).

2.2.1 Vitamin D: Introduction

Although it commonly referred to as a vitamin, vitamin D functions more accurately as a prohormone as it is synthesized endogenously in the skin through a photochemical reaction and acts as a signaling molecule which in turn influences various bodily functions. Upon exposure to ultraviolet B (UVB) radiation in the 290–315 nm range, the epidermal precursor 7-dehydrocholesterol, undergoes electrocyclization to form pre-vitamin D_3 which then spontaneously isomerizes into cholecalciferol (vitamin D_3), a compound structurally and functionally related to cholesterol (Feldman & Pike, 2011).

Cholecalciferol is transported in the circulation bound to vitamin D-binding protein and undergoes 25-hydroxylation in the liver to form 25-hydroxyvitamin D [25(OH)D], the principal circulating form and the standard biomarker of vitamin D status. It is further hydroxylated in the kidneys by 1α-hydroxylase (CYP27B1) to produce 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] (calcitriol), the hormonally active form. This final activation is tightly regulated by parathyroid hormone (PTH) in response to circulating calcium and phosphate levels (Cheng et al., 2004; Holick et al., 1995).

Vitamin D exerts broad neurophysiological and immunological effects in addition to its classical role in calcium-phosphate homeostasis and bone metabolism. Vitamin D receptors (VDRs) are widely expressed in the human brain, including neurons, astrocytes, and microglia, implicating it in brain-specific genomic regulation (Eyles et al., 2005). Moreover, extra-renal

synthesis of calcitriol by immune and glial cells suggests paracrine and autocrine signaling functions essential for neuroimmune crosstalk.

Functionally, vitamin D contributes to:

- Neuroprotection, through calcium regulation and antioxidant action,
- Cytokine modulation, promoting anti-inflammatory responses (e.g., IL-10, TGF-β1) and suppressing pro-inflammatory cytokines (e.g., TNF-α, IL-6),
- Neurotransmitter homeostasis, influencing glutamate, GABA, serotonin, and dopamine systems,
- Neurotrophic signaling, including upregulation of factors such as NGF and BDNF, Neuronal differentiation, plasticity, and synapse formation.

It is important to note that when serum 25(OH)D levels fall below 20 ng/mL (50 nmol/L), the body prioritizes its use for calcium regulation to maintain bone health. This physiological shift reduces the amount available for other critical functions, including neuromodulation and immune regulation, which are particularly important during prenatal and early postnatal brain development (Holick, 2005). This potential link forms the basis for exploring the mechanistic pathways through which vitamin D may influence ASD, as described in the following section.

2.2.2 Role of Vitamin D In Autism Spectrum Disorder: Gene Regulation and Neurodevelopmental Insights

Vitamin D may influence the development of Autism Spectrum Disorder (ASD) through two closely connected processes:

2.2.2.1. Regulating the activity of specific genes.

2.2.2.2. Supporting brain development, neural connectivity, and immune balance.

2.2.2.1 Gene Regulation

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 (calcitriol), binds to the vitamin D receptor (VDR) inside cells. This receptor–ligand complex attaches to defined regions of DNA, known as vitamin D response elements (VDREs), to activate or suppress the transcription of target genes (Ramagopalan et al., 2010). Many of these genes are critical for neural development, immune regulation, and the formation of synaptic connections (Freitag et al., 2010; Eyles et al., 2005).

Vitamin D also modulates epigenetic mechanisms, such as DNA methylation and histone modification, which influence long-term patterns of gene expression (Loke et al., 2015). Insufficient vitamin D during sensitive developmental stages may disrupt these genetic and epigenetic processes, increasing the likelihood of atypical brain development and ASD (Kalueff et al., 2006).

2.2.2.2 Brain Development and Immune Support

Beyond its genomic effects, vitamin D also plays a direct role in brain development and immune regulation. Both VDR and the enzyme required for calcitriol synthesis are detectable in the fetal brain by the first trimester (Cui et al., 2007; McGrath et al., 2001; Eyles et al., 2011). Adequate vitamin D supports neuronal growth, migration, and the establishment of stable synaptic connections.Its activity is particularly prominent in the hippocampus and cerebellum which are frequently implicated in ASD (Eyles et al., 2005; Taniura et al., 2006).

In addition, vitamin D enhances neurotrophic signaling, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and regulates neurotransmitters such as dopamine and serotonin, both of which are disrupted in ASD (Garcion et al., 2002).

On the immune side, vitamin D contributes to homeostasis by enhancing anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β1, while suppressing pro-inflammatory mediators (Cantorna et al., 1996; Garcion et al., 2002).In ASD, persistent neuroinflammation and microglial overactivation are commonly observed (Vargas et al., 2005). Suboptimal vitamin D status may intensify these immune disturbances, further impairing neural connectivity and function.

Taken together, these molecular and neuroimmune mechanisms provide biological plausibility for the hypothesis that vitamin D deficiency contributes to the pathophysiology of ASD (Eyles et al., 2005; Garcion et al., 2002; Vargas et al., 2005). The next section reviews observational evidence from serum studies that have sought to test this hypothesis in clinical populations (Mostafa & Al-Ayadhi, 2012; Bener et al., 2014; Wang et al., 2016).

2.2.3 Studies of Serum Vitamin D Levels in Autism Spectrum Disorder

2.2.3.1 Hypothesis Development from Observational Evidence

The conceptual foundation for this association was proposed by Cannell et al. (2008), who introduced the *"Vitamin D Hypothesis for Autism."* They argued that vitamin D insufficiency during pregnancy and early life could impair neurodevelopment through mechanisms such as

immune dysregulation, serotonergic dysfunction, and epigenetic modification. Building on this framework, subsequent observational studies have consistently reported that children with ASD have lower circulating 25(OH)D levels compared to neurotypical peers, with some studies also linking deficiency to greater symptom severity. Prenatal investigations further suggest that maternal deficiency confers elevated risk, highlighting a potential critical developmental window during gestation. Table 2.1 summarizes these findings across case–control, cross-sectional, and cohort studies**.**

Table 1: Summary of Key Studies on Serum Vitamin D Levels in Autism Spectrum Disorder

Authors(Year)	Study Design	Sample Size and Gender distribution (ASD= Male/Female; Control Male /Female)	Age Range (years)	Control Group Description	Key Findings	Conclusion
Mostafa & Al-Ayadhi (2012)	Case-control	ASD=40/10; Control: 24/6	3–10	Neurotypical children matched for age and gender	Serum 25(OH)D significantly lower in ASD; inverse correlation with CARS score	Vitamin D status may influence ASD symptom severity
Bener et al. (2014)	Case-control	ASD=60/20; Control: 58/22	3–11	Age- and sex-matched neurotypical children	ASD group had lower vitamin D and sunlight exposure; symptom severity inversely related to vitamin D	Environmental factors (e.g., sunlight) may contribute to vitamin D deficiency in ASD
Fernell et al. (2015)	Cross-sectional	ASD=Not specified; Control= Not specified	4–18	Age-matched neurotypical children	Lower vitamin D levels in ASD; correlation observed but causality not established	Confirms association but not causation between vitamin D and ASD
Zerbo et al. (2015)	Retrospective birth cohort	1,257 mother–child pairs (Mixed)	Maternal mid-gestation	Population-based maternal samples	Maternal 25(OH)D <20 ng/mL linked to higher ASD risk in offspring	Prenatal deficiency may increase neurodevelopmental vulnerability

Wang et al. (2016)	Cross-sectional	ASD: 57/10; No control group	3–8	No control group; reference ranges used	75% of ASD children had vitamin D insufficiency; lower levels correlated with CARS and ABC scores	Vitamin D status may affect behavioural phenotype in ASD
Ali et al. (2018)	Case-control	ASD: 32/8; Control: 30/10	4–12	Age- and sex-matched neurotypical controls	ASD group showed lower vitamin D, calcium, and magnesium	Broader micronutrient imbalance may contribute to ASD pathophysiology
Delhey et al. (2018)	Cross-sectional	ASD: 259/73; No control group	3–12	No control group; internal comparison by vitamin D status	35% vitamin D deficient; low levels linked to hyperactivity and irritability	Vitamin D insufficiency may exacerbate behavioural symptoms
Zhang et al. (2020)	Cross-sectional	ASD: 244/61; Control: 240/65	3–14	Age- and sex-matched healthy controls	Lower vitamin D and elevated PTH in ASD	Endocrine dysregulation may be involved in ASD

2.2.3.2 Meta-analytic Evidence on Vitamin D and ASD and Methodological Considerations

Meta-analyses of observational studies consistently demonstrate that children with autism spectrum disorder (ASD) have lower circulating vitamin D levels than neurotypical peers. A large synthesis of 34 studies including over 20,000 participants found that vitamin D was, on average, 7 ng/mL lower in children with ASD, with vitamin D deficiency conferring nearly a fivefold higher odd of ASD (Wang et al., 2021). Despite this clear directional trend, the analyses revealed substantial heterogeneity, suggesting that the strength of the association varies across regions and populations.

A pediatric-focused meta-analysis confirmed this finding, showing a moderate-to-large reduction in vitamin D levels among children with ASD. Importantly, it also examined symptomatology, reporting that lower vitamin D was associated with higher scores on clinical severity scales such as the Childhood Autism Rating Scale (CARS) and Autism Behavior Checklist (ABC). However, these associations weakened after adjustment for potential

confounders such as diet and outdoor activity, and inconsistencies across different severity measures limited the strength of conclusions (Gallardo-Carrasco et al., 2022).

Evidence from Observational cohort meta-analyses extend this association to early development. Prospective pooled analyses show that low maternal or neonatal vitamin D is associated with an approximately 1.5-fold higher risk of ASD in offspring, with stronger effects for maternal vitamin D (OR ≈ 2.7) than for neonatal levels (Wang et al., 2021). A recent dose–response analysis confirmed that higher maternal vitamin D levels during pregnancy were linked to progressively lower ASD risk, pointing to a potential prenatal window of vulnerability (Tirani et al., 2023).

While these findings are biologically plausible, several methodological challenges temper their interpretation. Meta-analyses consistently report substantial heterogeneity (I^2 often exceeding 90%), arising from differences in geography, seasonality, age groups, and laboratory assay techniques (Wang et al., 2021). Moreover, inconsistent cut-offs for defining vitamin D deficiency (<20 ng/mL vs. <30 ng/mL) alter pooled estimates and complicate comparisons across studies (Gallardo-Carrasco et al., 2022). The possibility of reverse causation has also been highlighted, as ASD-related behaviors such as reduced outdoor activity and selective eating may themselves contribute to lower vitamin D levels (Wang et al., 2021). In addition, residual confounding from unmeasured variables including BMI, nutrition, socioeconomic status, and comorbidities remains difficult to eliminate (Tirani et al., 2023). Associations with symptom severity are further weakened by the use of diverse assessment instruments (e.g., CARS, ABC, DSM-based measures), which contribute to variability in outcomes (Gallardo-Carrasco et al., 2022).

Overall, the literature suggests that vitamin D insufficiency is a consistent correlate of ASD, with some evidence for a modest prenatal risk effect (Wang et al., 2021; Tirani et al., 2023) and possible links to symptom severity (Gallardo-Carrasco et al., 2022). However, the strength of these associations remains inconsistent, largely due to methodological heterogeneity, confounding, and variability in outcome measures.

2.2.4 Vitamin D Supplementation as an Adjunctive Intervention in Autism Spectrum Disorder: Evidence from Interventional Trials

Vitamin D, a neuroactive steroid, has been increasingly investigated as a potential adjunctive therapy in autism spectrum disorder (ASD), owing to its roles in neuroimmune modulation, monoamine synthesis, synaptic plasticity, and gene regulation relevant to brain development