Original Article

DOI: 10.7860/JCDR/2025/76451.22114

Dentistry Section

Assessment of Salivary CD26/DPP-IV Levels and their Association with a Ketogenic Gluten-free Diet in Autism Spectrum Disorder: A Cross-sectional Study

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ABSTRACT

Introduction: Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition characterised by challenges in social interaction, communication, and the presence of repetitive behaviours. Gastrointestinal (GI) problems are frequently observed in patients with ASD, and dietary interventions, such as the Ketogenic Gluten-Free (KGF) diet, have been employed to manage these symptoms. Dipeptidyl-peptidase IV/CD26 (CD26/DPP IV), an enzyme expressed in the small intestine, plays an important role in protein digestion. Low levels of CD26/DPP IV have previously been reported to have implications in ASD.

Aim: To evaluate salivary CD26/DPP IV levels in children with ASD and compare them with healthy controls, considering KGF diet adherence.

Materials and Methods: The present cross-sectional study was conducted on children with ASD at the Arivu Early Intervention Centre for Special Children, Mangalore, Karnataka, India over one year (April 2021 to March 2022). The study group included children aged 3-14 years diagnosed with autism, selected using the Childhood Autism Rating Scale (CARS) criteria. The control group consisted of age- and sex-matched healthy children. Unstimulated saliva samples were collected from 42 participants (21 children with ASD and 21 healthy controls). CD26/DPP IV expression levels were estimated using Enzyme-Linked Immunosorbent Assay (ELISA). Information on GI symptoms and KGF diet adherence was obtained through a questionnaire completed by caregivers. Statistical analysis was performed using the Mann-Whitney U test to compare and correlate salivary CD26/DPP IV levels between groups. A p-value <0.05 was considered statistically significant.

Results: A total of 21 children with ASD and 21 age-matched neurotypical controls were included. The mean salivary CD26/DPP IV level was significantly lower in children with ASD (0.61±0.16 ng/L) compared to controls (0.88±0.25 ng/L; p<0.001). Within the ASD group, children adhering to a KGF diet showed slightly higher CD26 levels (0.56±0.17 ng/L) than those not on the diet (0.47±0.16 ng/L), and this difference was statistically significant (p=0.0113).

Conclusion: Salivary CD26/DPP IV levels were lower in children with ASD compared to healthy controls. Although children with ASD on a KGF diet showed slightly higher CD26/DPP IV levels, this difference was not statistically significant. Further studies are needed to establish the sensitivity and specificity of CD26/DPP IV and its potential role in early intervention for ASD.

Keywords: Children, Early detection, Enzyme Linked immunosorbent assay, Saliva

INTRODUCTION

Autism Spectrum Disorder (ASD) is a developmental disorder that affects communication. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), ASD is characterised by deficits in two major areas: social interaction and communication. It is also associated with restricted, repetitive, and stereotypical behaviours [1]. Autism is referred to as a "spectrum condition" because of the wide variation in the type and severity of symptoms. The World Health Organisation (WHO) reports that ASD is prevalent in 0.76% of the global population, of which 16% are children [2]. In India, out of a total population of 1.3 billion, nearly one-third are children aged ≤15 years. Based on recent studies, it is estimated that around two million people in India may be affected by this condition [2].

Both genetic and environmental factors have been reported to contribute to the development of ASD [3]. Prenatal infections with viruses such as rubella and cytomegalovirus (CMV), as well as exposure to heavy metals, have been linked to autism in some cases. However, the aetiology of ASD remains unclear. Children with ASD are also more likely to have genetic syndromes such as Fragile X syndrome, neurofibromatosis type 1, Down syndrome, and tuberous sclerosis complex. Neurological disorders, including epilepsy and cerebral palsy, as well as congenital abnormalities of the nervous system, are more common in children with ASD compared to neurotypical children [4]. Most children with ASD present with gastrointestinal (GI) disorders, which usually manifest as feeding problems such as food selectivity, food refusal, and poor oral intake, compared to neurodevelopmentally normal children [5].

Dipeptidyl-peptidase IV (DPP-IV/CD26) is a cell-surface protease belonging to the prolyl oligopeptidase family. It is expressed in the cells lining the villi of the small intestine and functions in breaking down small peptides into di- and tripeptides that can be transported across the intestinal wall. It also plays an important role in the digestion of casein-containing foods [6]. Deficiency of the DPP-IV enzyme results in maldigestion of these dietary proteins and the production of small peptides, which can bind to opioid receptors in the brain, affecting cognitive function and contributing to the aggressive behaviour observed in some patients with ASD [7].

By eliminating gluten and casein, the Ketogenic Gluten-Free (KGF) diet reduces the accumulation of undigested peptides, thereby potentially mitigating gut-brain axis dysfunction and behavioural issues in ASD [8].

Currently, treatment options for symptomatic relief in ASD are limited [9]. Dietary interventions have been employed as an alternative

approach to managing neurodevelopmental disorders. A Ketogenic Gluten-Free (KGF) diet consists of high fat (80%), moderate protein (15%), and very low carbohydrate content (5%), with a lipid-to-non-lipid ratio of 4:1. This forces the body to utilise fat as the primary source of energy [10]. The inflammatory response caused by casein maldigestion can alternatively be managed with supplemental DPP-IV, in place of strict dietary restrictions [11].

Dietary factors may influence the behaviour, mood, and cognition of children with ASD due to alterations in the intestinal microbiome [12]. It is hypothesised that a KGF diet may help increase CD26/DPP-IV levels in children with ASD, whereas the null hypothesis states that a KGF diet will not increase CD26/DPP-IV levels in this cohort. To date, no studies on salivary CD26/DPP-IV levels in Indian children have been reported in the literature. Therefore, this study aimed to evaluate alterations in salivary CD26/DPP-IV levels and to analyse the influence of both CD26/DPP-IV levels and KGF diet adherence in children with ASD in an Indian cohort.

The primary objective of this study was to estimate salivary CD26/DPP-IV levels in children with ASD compared to neurotypical controls. The secondary objective was to assess changes in CD26/DPP-IV levels associated with KGF diet adherence among children with ASD.

MATERIALS AND METHODS

The present cross-sectional observational study was conducted at the Arivu Early Intervention Centre, located in Mangalore, Karnataka, India. The study was carried out over a period of 12 months, from April 2021 to March 2022. Ethical clearance for this study was obtained from the Institutional Ethics Committee (IEC) with approval number YEC2/594. Written informed consent was obtained from the parents or legal guardians of all participants prior to sample collection.

Sample size calculation: The sample size was calculated based on a previous study investigating serum biomarkers in children with ASD. The mean (SD) of CD26/DPP-IV in the autistic and control groups were 0.69 (0.08) and 1.07 (0.05), respectively [13]. The corresponding effect size was 5.7. For calculation, a large effect size of 0.8 was considered. The sample size was determined using G*Power software. To detect the anticipated difference between the control and autism groups at a 5% level of significance and 80% power, 21 subjects were required in each group. The total sample size was therefore 42. Among the 21 children with ASD, 7 followed the KGF diet while 14 did not. All eligible children present during the study period were included.

Inclusion criteria:

- Children aged 3 to 14 years diagnosed with ASD, assessed using the Childhood Autism Rating Scale (CARS) [14].
- Controls: Age- and sex-matched neurotypical children with no history of developmental disorders.

Exclusion criteria:

- Children with other neurological or metabolic disorders.
- Children who had taken antibiotics, immunosuppressants, or probiotics within the four weeks prior to sampling. Children with systemic illnesses affecting saliva production.
- Children with systemic illnesses affecting saliva production.

Study Procedure

- A structured questionnaire was used to collect demographic details (age and gender) and the presence of GI symptoms [Annexure 1].

Saliva collection: Passive drool samples were obtained between 10 AM and 2 PM by asking participants to pool saliva in their mouths and deposit it into a sterile polypropylene culture tube (12×75 mm, 5 mL capacity). In challenging cases, a sterile absorbent cotton roll was placed in the mouth until saturated (approximately 30-40 seconds) and then deposited back into the container. Research staff supervised each participant individually to ensure protocol adherence and adequate sample collection. They ensured that participants did not touch the saliva or related materials and that samples were appropriately stored.

To prevent contamination, participants were instructed to avoid eating a major meal within 60 minutes prior to collection. They were also advised to avoid foods high in sugar, acid, or caffeine immediately before sample collection, as these could lower salivary pH and increase bacterial growth, potentially compromising the assay.

The saliva containers were placed in an ice box and transferred to the laboratory. Samples were centrifuged to remove unwanted particulates, and the supernatant was separated and stored at -80 °C until biochemical analysis (ELISA) was performed.

The stored centrifuged saliva samples were analysed using a CD26/DPP-IV ELISA kit to detect and quantify enzyme concentration based on antigen-antibody binding. Absorbance values were read using a spectrophotometer, and CD26/DPP-IV levels were compared between the groups.

STATISTICAL ANALYSIS

All statistical analyses were carried out using GraphPad Prism 7. The Mann-Whitney U test was used to compare salivary CD26/DPP-IV levels between groups and controls for each clinicopathological parameter. A two-tailed p-value<0.05 was considered statistically significant.

RESULTS

This study was designed to estimate and compare salivary CD26/DPP-IV levels in 21 children with ASD and 21 healthy controls using the ELISA method, and to evaluate the effect of adherence to a Ketogenic Gluten-Free (KGF) diet.

The total sample size was 42, of which 30 (71%) were males and 12 (28.5%) were females. Among the 21 children with ASD, 16 (76%) were males and 5 (24%) were females. The age of participants in the ASD group ranged from 6 to 14 years, with a mean age of 10 years. The control group consisted of age-matched healthy children with the same mean age [Table/Fig-1].

Study group	Total participants	Total male participants	Total female participants	Age range (in years)	Mean age (in years)
ASD	21	16	5	6-14	10
Control	21	15	6	6-14	10

[Table/ Fig-1]: Mean age and sex in ASD and normal groups.
All values are presented as mean, standard deviation, test statistics, and p-value. CD26/DPP-IV values are reported in ng/L. Group 1: Children with ASD (N=21); Group 2: Healthy controls (N=21). Statistical test used: Mann-Whitney U test; significance threshold: p<0.001.

The salivary CD26/DPP-IV concentration in the ASD group (Group 1) ranged from 0.274 ng/L to 0.887 ng/L, with a mean value of 0.61 ng/L. In the control group (Group 2), the concentration ranged from 0.601 ng/L to 1.383 ng/L, with a mean value of 0.88 ng/L. A statistically significant difference was observed between the two groups (p<0.001) [Table/Fig-2]. Furthermore, salivary CD26/DPP-IV concentrations were compared between children with ASD who followed the KGF diet and those who did not. In the diet group, concentrations ranged from 0.437 ng/L to 0.992 ng/L, with a mean of 0.56 ng/L. In the non-

	Group	N	Mean	Standard Deviation	Mann-Whitney U Test statistics	p-value
Concentration of CD26 (ng/mL)	ASD (Group 1)	21	0.61	0.16	-3.663	<0.001
	Control (Group 2)	21	0.88	0.25		

[Table/Fig-2]: Comparison between salivary CD26/DPP IV of control and salivary CD 26/DPP IV of ASD.

diet group, concentrations ranged from 0.274 ng/L to 0.641 ng/L, with a mean of 0.47 ng/L. A statistically significant difference was observed between these subgroups (p=0.0113) [Table/Fig-3].

Type of diet	Mean	Std. Deviation	Percentiles			Mann-Whitney U Test statistics	p-value
			25	50	75		
KGF diet (n=7)	0.56	0.17	0.47	0.57	0.72	-1.59	0.0113
Without KGF diet (n=14)	0.47	0.16	0.30	0.47	0.64		

[Table/Fig-3]: Comparison of salivary CD26/DPP IV levels in ASD children following Ketogenic Gluten Free (KGF) diet and those not following the Ketogenic Gluten Free (KGF) diet.
All values are presented as mean, standard deviation, percentiles, test statistics, and p-value. CD26/DPP-IV values are reported in ng/L. Group 1: Children with ASD on KGF diet (N=7); Group 2: Children with ASD without KGF diet (N=14). Statistical test used: Mann-Whitney U test; significance threshold: p=0.0113.

DISCUSSION

Currently, there are no specific diagnostic tests or biomarkers for the early detection of autism [11]. Therefore, there is an urgent need to develop reliable biomarkers to improve prognosis in children with ASD. Biomarkers are objective indicators of biological or pathophysiological processes, as well as pharmacologic responses to therapeutic interventions [12]. They have the potential to be applied in multiple aspects of clinical care for patients with ASD, including early diagnosis and treatment selection [15].

CD26/DPP-IV is a multifunctional type II cell surface glycoprotein expressed on T cells, B cells, Natural Killer (NK) cells, and on epithelial, endothelial, and acinar cells in various organs [16]. CD26/DPP-IV levels are often reduced in inflammatory and autoimmune conditions because CD26 plays an important role in T cell activation, proliferation, and differentiation [17].

There is a paucity of studies using saliva as a diagnostic medium for ASD. Saliva has not been routinely evaluated in clinical laboratories for this purpose. In this study, a significant decrease in salivary CD26/DPP-IV concentrations was observed in children with ASD compared to healthy controls.

Previous studies examining plasma and serum levels of CD26/DPP-IV in healthy and autistic individuals reported results similar to the salivary values obtained in this study [13,18]. In a study by Ratajczak HV et al., CD26/DPP-IV levels in neurotypically healthy autistic adults and controls were also consistent with our findings [19]. Variations between studies may be attributed to differences in methods used for estimating CD26/DPP-IV levels [13,20]. Notably, no studies have evaluated salivary CD26/DPP-IV levels in Indian children with ASD to date.

A significant reduction in CD26 expression was observed in children who did not follow the KGF diet compared to those who did, supporting the study hypothesis. The inclusion of a control group helped establish baseline salivary CD26/DPP-IV levels, allowing meaningful comparison with the ASD group. These results align with the study by Alaamey et al., which reported significantly lower serum DPP-IV activity in ASD children not following a specific diet ($p<0.05$), demonstrating consistency across serum and saliva samples [20]. CD26 is a protein-digesting enzyme, and reduced expression may interfere with cognitive function. Therefore, a highly restricted diet regimen, combined with CD26/DPP-IV supplementation, may be beneficial in improving symptoms in children with ASD [13,20].

Relatively few biomarkers have been assessed in saliva from autistic individuals, as urine or blood are generally preferred. However, collecting blood or urine samples from children requiring special care can induce anxiety and stress [21,22]. Therefore, selecting a body fluid with minimal collection-related stress is crucial to avoid altering biomarker levels. Salivary biomarker concentrations are expected to be lower than those in blood or urine, as saliva is considered a filtrate of the blood [23,24]. The extended range of ELISA standard curves allowed measurement of these lower concentrations. Saliva was chosen for this study because CD26/DPP-IV is present in detectable amounts and was found to be significantly decreased in children with ASD.

Limitation(s)

The relatively small sample size reduces the statistical power and limits the generalisability of the findings to the broader population of children with ASD. The cross-sectional study design restricts the ability to establish causal relationships between salivary CD26/DPP-IV levels, dietary adherence, and GI symptoms. Additionally, potential confounding factors—such as variations in dietary compliance, gut microbiota composition, medication use, and comorbid conditions—were not fully controlled, which may have influenced the observed outcomes.

CONCLUSION(S)

This study estimated salivary CD26/DPP-IV levels in children with ASD and healthy controls. The findings revealed that salivary CD26/DPP-IV levels were significantly lower in children with ASD compared to healthy controls. Furthermore, children with ASD who did not adhere to a KGF diet had significantly lower CD26/DPP-IV levels than those who did. These results highlight the potential of saliva as a non-invasive diagnostic medium for facilitating early intervention in ASD. However, further research with larger cohorts is needed to validate these findings.

Acknowledgement

We would like to thank the Indian Council of Medical Research (ICMR) (MD21JUN-00092) for funding and supporting this project. We also extend our gratitude to the Arivu Early Intervention Centre for Special Children, Sakthi Nagar, Mangalore, for their collaboration, which made this study possible.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Oct 27, 2024
- Manual Googling: Jul 21, 2025
- iThenticate Software: Jul 23, 2025 (10%)

ETYMOLOGY: Author Origin

EMENDATIONS: 8

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Oct 26, 2024**
Date of Peer Review: **Jan 18, 2025**
Date of Acceptance: **Jul 25, 2025**
Date of Publishing: **Dec 01, 2025**

STRUCTURED QUESTIONNAIRE FOR PARENTS/CAREGIVERS- [ANNEXURE I]

This form helps us understand your child's diet and any tummy issues they might have. Please fill this to the best of your knowledge.

What is a KGF Diet?

A Ketogenic Gluten-Free (KGF) Diet is a special diet where:

☑ All gluten (wheat, barley, rye) is removed from the food.

☑ The diet also has more healthy fats and fewer carbohydrates (like rice, bread, sugar).

☑ Some children with autism follow this diet to help with their digestion and behaviour.

Section 1: Child's Basic Details

Child's Name: _____

Age: _____ years

Gender: ☐ Boy ☐ Girl ☐ Other

Section 2: About Your Child's Diet

Is your child currently following the special Ketogenic Gluten-Free (KGF) diet?

☐ Yes (Please go to Question 5) ☐ No (Please go to Question 8)

Who suggested this diet?

☐ Doctor/Nutritionist ☐ Other parents ☐ Online research ☐ Family decision

How long has your child been following the KGF diet?

Since: _____ (Month/Year)

How strictly do you follow the KGF diet?

☐ Very strict - We follow it for all meals/snacks every day

☐ Mostly strict - We follow most of the time, but some exceptions happen

☐ Not strict - We follow it only sometimes

Section 3: For Children NOT Following the KGF Diet

What type of diet does your child eat every day?

☐ Regular Indian home food (includes chapati, bread, biscuits, etc.)

☐ Vegetarian diet ☐ Non-vegetarian diet

☐ Other (please describe): _____

Section 4: Stomach Problems (GI Symptoms)

In the past 6 months, has your child had any of these tummy problems? (Tick all that apply)

☐ Constipation (trouble passing stool)

☐ Loose motions/diarrhea

☐ Stomach pain

☐ Bloating (tummy feels full or tight)

☐ Other (please describe): _____

How often does your child have these tummy problems?

☐ Daily ☐ Once a week ☐ Once a month ☐ Occasionally

Do these problems get better when you change your child's diet (like stopping gluten or starting KGF diet)?

☐ Yes ☐ No ☐ Not sure

Section 5: Any Other Important Information

Is your child taking any special foods, vitamins, or medicines daily?

☐ Yes (please list them): _____

☐ No

Signature of Parent/Caregiver: _____

Date: _____