# Salivary Factors Related to Caries in Children with Autism

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*Many predisposing factors to caries are present in autism, however, it is unlikely that autistic patients exhibit higher caries indexes than the rest of the population.* ***Objective:*** *To evaluate salivary factors related to caries in autistic patients.* ***Study design****: 34 autistics and 34 controls aged between 4-13 years old were included. Decayed, missing, and filled teeth (DMFT) index and oral hygiene simplified index (IHO-S) were assessed, as well as, pH, total proteins, phosphate, calcium and IgA in saliva. All data were analyzed by chi² and Student t tests for independent samples. P values<0.05 were considered statistically significant.* ***Results:*** *Autistic patients showed less caries than controls (p≤0.001), DMFT was 1±1 and 3±2 respectively (p≤0.001). In relation to IHO-S, values increased (p=0.008) in autistic patients (2.25±0.78) compared to controls (1.79±0.59), however Salivary ph means were similar (7.20±0.48 and 7.27±0.34 respectively). Decreased calcium levels (p=0.013) were observed in autistics (0.621±0.35 mmol/L) compared to controls (0.89±0.51 mmol/L), but phosphate levels were similar (6.17±4.22 M, 5.51±4.86 M respectively). When total proteins of saliva were assessed, autistics showed a slight increment (2.65±1.81 mg/mL) compared to controls (2.24±1.27 mg/mL) and zymography showed a higher proteolytic activity in autistic children. Finally, IgA concentration reached 116.55±90.97 µg/mL in autistics and 161.61 ± 193.37µg/mL (p=0.527) in the control group.* ***Conclusions****: Even though patients with autism exhibited a poorer oral hygiene, caries indexes were lower, calcium levels in saliva were found to be lesser and phosphate levels higher.*

*Keywords: Autism, saliva, caries ,children,*

## INTRODUCTION

Autism is a serious and complex disorder first described in 1943 by Kanner based on brain development [1]. The main characteristics of autism are related to socialization issues, communication deficit, repetitive and stereotyped behavior and cognitive inflexibility. There is no biochemical marker that allows diagnosing autism, therefore, it is identified based on the patient´s clinic and behavior [2].

In relation to the severity of autism, it has been classified in grades depending on dimensions within the spectrum. For instance, grade 1 refers to a mild deficit and grade 3 the most severe level of nonverbal communication skills [3].

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There are some clinical conditions present in autism, such as gastrointestinal problems, disorders in neurological development affecting brain function, high prevalence of epilepsy in up to 46% of patients and sleep disorders, such as insomnia or apnea [4]. Due to such disability to digest certain proteins, Defeat Autism Now (DNA) protocol is indicated in patients with autism. This is a biological and nutritional treatment proposition published for the first time in 1996 [5]. The main goal of this treatment is to help the organism to detox. In this protocol the diet must be free of gluten, casein, additives and refined sugar [6,7], elements associated to dental caries.

Oral health of these patients has also specific characteristics. Children with autism prefer soft foods and this makes them more susceptible to dental caries. Additionally, behavior disorders usually render oral hygiene and dental attention more difficult in this type of patients [8]. Despite these conditions which favor the development of caries, it is reported that autistic children do not evidence a higher caries index, considering all their predisposing factors. Therefore, there is a growing interest in the diagnosis through the saliva in relation to the potential benefits to prevent dental caries [9]. The objective of this study was to evaluate salivary factors related to caries in autistic patients.

## MATERIALS AND METHOD

68 male patients were selected. 34 children had been diagnosed with autism, compared to 34 healthy controls( ± 2 years). Autistic patients were selected from 2 schools for patients with autism in the city of Caracas, and healthy patients were selected from private schools in the same city. All those patients with grade 1 and 2 diagnosed autism were included.

Children with GI disorders, such as gastroesophageal reflux altering oral pH, patients consuming drug which might cause xerostomia or produce gingival hyperplasia, patients with concomitant syndromes, such as Down syndrome and patients with autism grade 3 were excluded.

Bioethical approval was granted by the Bioethics Committee of the Faculty of Dentistry of Universidad Central de Venezuela (Number 0421-2013). All parents were informed in detail about the study and consent was signed for approval of participation.

All patients were evaluated using artificial light and a clinical dental mirror. The presence of caries, gingivitis and dental plaque were measured. In this procedure the number of affected teeth was analyzed through decayed, missing and filled teeth index (DMFT) under World Health Organization 1997 criteria (10). Also, the Simplified Oral Hygiene Index (IHO-S) was measured in six teeth's surfaces with a score from 0 to 3 to determine the presence of debris [11]. All the patient's parents were asked about the diet of the children, if they follow or not a diet free of gluten, casein and salicylates.

### Recollection of saliva and pH measurement

After the clinical exam a 5 cc of unstimulated whole saliva sample was collected using sterile disposable cups. Patients were told not to eat or drink two hours before the sample was taken. pH was measured using a Hanna Piccolo manual pH meter, previously calibrated with pH 4 and 7 standards. Then, the sample was cryopreserved and taken to the laboratory and centrifuged at 16000 g for 10 minutes to remove unwanted debris.

### Calcium, Phosphate and Proteins assessment

Calcium content in the saliva sample was estimated by the colorimetric method of Connerty and Briggs [12] using O-cresolphthalein complex. Absorbance was measured at 570 nm in any suitable spectrophotometer or colorimeter. Phosphate was determined by the Fiske and Subbarow [13] method which is based on the ready solubility of the reducible phosphomolybdic acid in isobutyl alcohol. It consists essentially of the reduction of phosphomolybdic acid to the blue complex by shaking the alcoholic extract with an acidified aqueous solution of stannous chloride. Finally, total proteins were estimated by Folin Ciocalteu method and absorbance was measured at 750 nm after 30 minutes [14].

### Polyacrylamide gel electrophoresis and zymography for protein evaluation

Salivary proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (10% separating gel, 5% stacking gel) according to the method of Laemmli [15]. Zymography was performed for the detection of hydrolytic enzymes, based on the substrate repertoire of the enzyme. Gelatin embedded in a polyacrylamide gel was digested by active gelatinases run through the gel. After Coomassie staining, areas of degradation were visible as clear bands against a darkly stained background [16].

### IgA concentration measurement

The determination of total salivary IgA was performed by ELISA modified method and the absorbance was measured at 492 nm [17].

## RESULTS

### Caries and IHO-S indexes

In the group of patients with autism, 79.41% (27 patients) were caries free. Contrary, 73.52% (25 patients) of the control group were diagnosed with caries. Patients with autism exhibited less percentage of caries which was statistically significant ($p \leq 0.001$). DMFT index in autistic patients was 1 ± 1 and controls 3 ± 2. Patients with autism showed a statistically significant lesser caries index ($p \leq 0.001$).

In terms of oral hygiene, children with autism showed more dental plaque than controls (64.70% vs 61.80%), however it was no statistically significant ($p = 0.042$). IHO-S index was 2.23 ± 0.83 in autistic children while it was 1.82 ± 0.60 in healthy children. Patients with autism exhibited a statistically significant ($p=0.008$) poorer oral hygiene.

### Diet

In the group of the 34 autistic patients, 18 (52.94%) followed a diet free of gluten, casein and salicytates and 16 (47.05%) did not have any specific diet. Presence of caries was similar regardless of the diet ($p=0.810$).

### Saliva pH

Although, pH of study group was slightly lower compared to controls (7.17±0.45 and 7.27±0.28 respectively), there was no statistically significant difference between the samples ($p=0.497$).

### Calcium and phosphate levels in saliva

The group of patients diagnosed with autism exhibited a calcium mean of 0.621±0.353 mM, vs control patients with a mean of 0.897 ± 0.518 mM. Patients with autism showed statistically lower calcium levels ($p=0.013$). Likewise, it was determined that calcium levels in autistic patients with special diet (0.51 mmol/L ±0.29) were lower than those not following the diet (0.74 mmol/L ± 0.38).

Regarding phosphate levels, autistics showed an increase in mean values compared to the control group (6.17 ± 4.22 M and 5.51 ± 4.86 M). Even though the mean in autistic patients was higher, there was not statistically significant difference ($p = 0.738$). When group was split based on the presence of caries, highest values were found in the group of autistic children with caries (8.91±5.60 M) vs those who did not exhibit caries (5.25±3.60 M). Similar levels of phosphate were observed in the control group regardless of the presence of caries (5.10±7.06 M control group with caries and 5.78±3.52 M control group caries free).

### Protein profile

A higher amount of salivary proteins were found in autistics (2.65±1.81mg/mL) compared to control group (2.24±1.27 mg/mL), however no statistically significant ($p=0.280$). In Figure 1, the results of polyacrylamide gel electrophoresis are observed. Medium molecular weight proteins were more abundant in patients with autism.

There were important differences in the proteolytic pattern between both groups of patients (Figure 2). A more intense proteolytic activity was observed in autistic gels, in which metalloproteases

had a larger activity, both in high molecular weight proteins, which ranged between 267.90 and 163.10 KDa, as well as in medium and low molecular weight, from 130.52 KDa.

Finally, it was observed that the group of patients with a diagnosis of autism exhibited a mean IgAs of 116.55 µg/ml ± 90.97 vs control patients with a media of 161.61 µg/ml ± 193.37. Even though the median in autistic patients was lower, no statistically significant difference was observed ($p = 0.527$). When IgAs values were compared in both groups of patients with and without caries, higher IgAs levels were observed in autistic patients without caries, as well as in control patients with caries. Table 1 shows the summary of the results.

**Table 1. Summary of Results**

| | Autistic | Control | p<0,05 |
|---|---|---|---|
| Presence of Caries | 20.60% | 73.50% | ≤0.001 |
| Presence of plaque | 64.70% | 61.80% | 0.80 |
| Caries Index | 1 | 3 | ≤0.001 |
| IHOS | 2.2 5 | 1.79 | 0.008 |
| Salivary pH | 7.20 | 7.27 | 0.49 |
| Salivary Calcium | 0.62mmol/L | 0.89 mmol/L | 0.01 |
| Salivary phosphate | 6.17 M | 5.51 M | 0.73 |
| Salivary IgA | 117 µg/mL | 162 µg/mL | 0.52 |
| Total Protein Concentration | 2.65 mg/mL | 2.24 mg/mL | 0.28 |

**Fig 1. 10% polyacrylamide gel electrophoresis (9 X 14 cm) dyed in Coomassie Blue. A: Electrophoretic pattern in saliva of control patients (lane 1–4) and autistic patients (lanes 1'–4'); the standard of the molecular mass (kDa) is observed to the left; the bands are observed to the right. B: Correspondence of the observed bands with salival proteins identified and reported. CP (Control Patient); AP (Autistic Patient). The figure in parenthesis is the location of the lane at the gel of Figure A. C: Molecular mass of each band.**

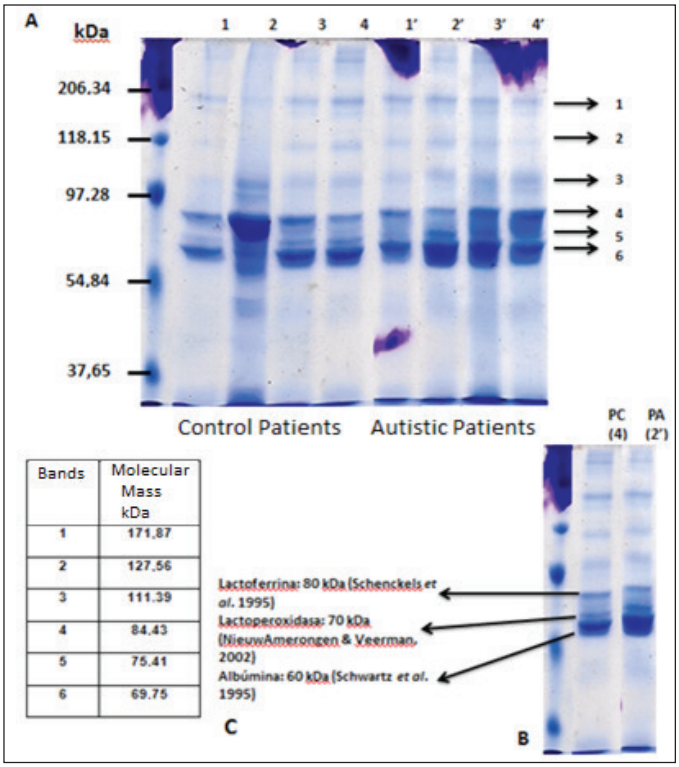
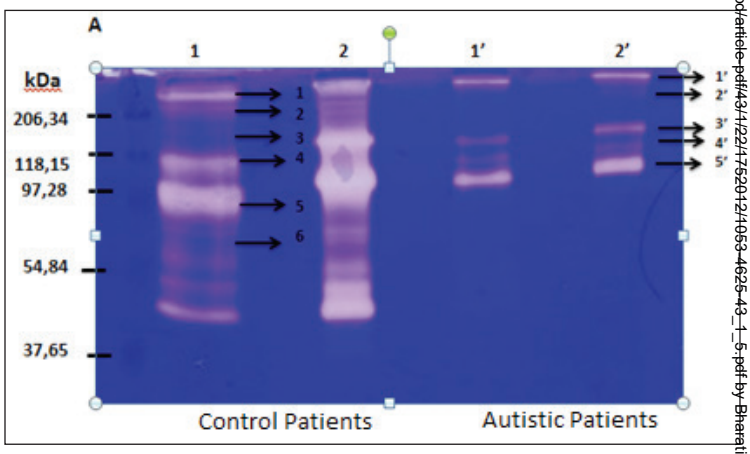



**Figure 2. 10% Polyacrylamide gel electrophoresis (9 X 14 cm) dyed in Coomassie Blue (Zymography). A: Electrophoretic pattern in saliva of control patients (lane 1 and 2) and autistic patients (lanes 1'–2'); the standard of molecular mass (kDa) is observed to the left, the bands are observed to the right. B: Molecular Mass of each band in both groups.**




B

| Bands | Molecular Mass kDa |
|---|---|
| 1 | 216.70 |
| 2 | 193.83 |
| 3 | 160.29 |
| 4 | 133.13 |
| 5 | 110.17 |
| 6 | 88,28 |

| Bands | Molecular Mass kDa |
|---|---|
| 1' | 267,90 |
| 2' | 227.36 |
| 3' | 163.10 |
| 4' | 145.22 |
| 5' | 130.52 |

## DISCUSSION

The process of dental caries involves different factors, such as diet, hygiene, host susceptibility, oral microbiota, and salivary factors, among others. Patients with autism usually have some factors that increase the risk of developing caries, such as the medications received, preference of soft food, fluoride contraindication, calcium free diet and poor oral hygiene due to low skills to brush their teeth [18]. In this study, a lower rate of dental restorations and caries was determined in a population of autistic patients. These results evidence that, despite all predisposing factors autistic patients are exposed to, intrinsic factors with impact in lower dental caries prevalence.

Decreased caries prevalence in autistic population has been reported previously. Vajawat *et al* [19], Marshall *et al* [20] and Al-Maweri *et al* [21] studied different groups of autistic patients and healthy individuals, observing a lower DMFT in autistic patients than in the control subjects and therefore lower caries prevalence.

The results of this study evidences that there is deterioration in oral hygiene in autistic patients as they showed more detritus and dental plaque or biofilm. In fact, it was determined that oral hygiene of autistic patients was poor compared to control group, which is similar to the results obtained by Rai *et al.* [9]. Therefore, it is important to stress that despite dental biofilm being a basic factor in caries development; it was not enough for caries increment in autistics. Hence, there might be a protective factor, probably in saliva, which may offset all cariogenic risk factors these patients are exposed to.

Regarding salivary factors, pH in autistic patients was more acidic and variable, but since differences between controls were very low, it was not considered an influencing factor on the caries index of autistic population. Bassoukou *et al* , and Rai *et al* [22,9] conducted similar studies and did not find any statistically significant differences nevertheless, it is always considered in the studies because saliva buffering capacity works by counteracting the decrease in pH and is an important factor protective against caries.

Interestingly, calcium and phosphate in the saliva of autistic were inversely proportioned. As calcium was decreased, higher phosphate levels were observed. This can be related to the diet followed by most autistic children, which is gluten and casein free. It is probable that, since dairy consumption is low, there is a decline in calcium levels, given that autistic patients who followed a special diet show even lower levels of calcium.

For children, milk can represent 75% of the total calcium diet intake. Therefore, in these patients, calcium had a weak correlation with caries activity. Hence it can be speculated that it is not the factor that is protecting them from dental caries; since it should be more saturated in saliva to become an active factor of remineralization process. Indeed, prior studies have found lower levels of urine and hair calcium in autistic patients, which could be related with fat malabsorption as well, which is part of the mechanism limiting dietary calcium reabsorption [23,24].

On the other hand, phosphate levels were higher in autistic patients. This might be a key point concerning caries levels in autistics, since phosphate plays an important role in salivary buffering capacity and in remineralization process [25]. In the same vein, autistic patients have higher levels of biofilm, which contains two to three times more phosphate than saliva. However, the biofilm and saliva are in a constant ionic exchange, so the saliva of these patients could have more abundant levels of phosphate that would come from such exchange. That is to say, at the moment of initiating the carious process in these patients, the phosphate rises, coming from the biofilm to initiate the process of remineralization and to try to stop the advance; This may be a protective factor in these patients and this is why they have less caries and progress slower than in the control group [26].

Vijayashankar *et al* [27] performed an analysis of saliva proteins from autistic patients and healthy individuals concluding that there is an abnormal increase of proteins with a molecular weight between 52 and 63 kDa. In fact, the authors state some proteins might serve as markers of autism. Likewise, the authors observed a decrease of proteins with molecular weights between 63 and 76 kDa, which contrast with the findings of this investigation.

Interestingly, important differences in protein saliva profile of autistics were observed. Proteins around 70 kDa were more abundant in autistic patients, suggesting lactoperoxidase, a low molecular weight protein of 73-78 kDa, may be increased. Lactoperoxidase comprises antibacterial properties; it catalyzes the products oxidation, which could react rapidly with sulfhydryl groups of bacterial enzymes involved in obtaining energy from glucose, inhibiting its function and acids production. Another function not related to the generation of oxidizing agents is the inhibition of extracellular polysaccharides production which strengthens the attachment of bacteria to the dental surface in the biofilm. This would explain the important role in dental protection against caries [28].

In relation to zymography, a higher proteolysis pattern was observed in autistic patients vs the control group. This could be due to the increase of metalloproteinases MMP-1, MMP-3 and MMP-9 which usually increase in healthy but swollen gums as a result of biofilm presence, which is very common in autistic patients because of their poor hygiene [29].

When salival IgAs levels were studied, lower levels were observed in autistic patients. This might be correlated with the immunological deficiencies related to this condition, which have been described within the alterations of the immunologic system in autistic patients with low IgA levels and high IgE levels [30]. However, when IgA levels are observed in autistic patients with and without caries, it was determined such levels were higher in autistic patients without caries. These results are similar to those obtained by Doifode *et al*, Chawda *et al* [31,32] who after studying a group of patients with and without caries found higher values in patients free of caries. Both results may be explain due to higher IgA levels which protect against caries.

## CONCLUSIONS

- Patients with autism exhibited lesser caries prevalence and lower caries indexes than control patients.
- Phosphate and secretory IgA levels play an important role in the protection against caries in autistic patients.
- The protein profile showed proteins of a molecular weight of around 70 KDa, probably lactoperoxidase, which might be accomplishing a bactericidal role.
- Some salivary factors might represent a protection against caries even with unfavorable oral conditions in autistic patients

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