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Association Study between DUF1220 Copy Number and Severity of Social Impairment in Sex-balanced Simplex Cases of Autism

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ABSTRACT

Introduction: Copy number variations (CNVs), which are genetic factors responsible for human evolution, have emerged as underlying pathogenic factors for a number of diseases such as autism spectrum disorders (ASD). DUF1220 coding sequences have been shown to be positively associated with the severity of symptoms in familial/multiplex cases of autism. However, this association has not been confirmed in simplex autism, and the potential impact of gender/sex has not been studied.

Methods: Using saliva samples taken from Iranian children with non-syndromic simplex autism, different ethnicity/race and genetic backgrounds from previous studies, we assessed the association between DUF1220 CNVs and Autism Diagnostic Interview-Revised (ADI-R) domain scores in both males and females.

Results: In the male and female combined group with autism, in line with previous reports, our findings showed that there were no significant associations between DUF1220 CNVs with either total ADI-R score, social, communication, or repetitive diagnostic scores in simplex autism cases. Interestingly, however, in sex classified groups, despite the insignificant results, our findings in girls with autism showed a negative trend between DUF1220 CNVs and severity of symptoms for the social interaction and communication domains. By contrast, in male children with autism, the results showed a positive trend.

Conclusion: It seems that association of DUF1220 CNV with the severity of symptoms in simplex children with autism may follow a sexually dimorphic pattern that needs to be re-examined in prospective studies.

Keywords: Autism spectrum disorders, copy number variation, DUF1220, simplex autism, sex balance

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INTRODUCTION

Copy number variations (CNVs) are structural variations, characterized by deletion or duplication in the number of copies of specific DNA regions and are the underlying factor in human evolution and diseases (1,2). CNVs have emerged as pathogenic factors for many psychiatric disorders, including autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and intellectual disability (ID) (1,3,4). Childhood autism, the most severe subset of ASD, is a neurodevelopmental and lifelong condition that usually appears in the first three years of life and is characterized by deficits in social interaction and communication, and by restricted and repetitive patterns of behavior (5). The cause of autism is not fully known. However, genetic factors play a major role in the pathophysiology of autism. A recent study of familial risk estimated ASD heritability at 83% (6). The genetic anomalies leading to ASD are heterogeneous and range from high penetrating single-gene mutations and CNVs to weak penetrating risk alleles (7). Furthermore, it has been estimated that about 30%-39% of all cases have *de novo* mutations, a good number of which are related to rare CNVs (8).

Highlights

- **The impact of sex in Autism Spectrum Disorders (ASD) remains largely unexplored.**
- **DUF1220 CNV was previously associated with ASD core phenotypes in multiplex cases.**
- **We found no such significant association in sex combined simplex autism cases.**
- **Male and female simplex cases of autism, however, may follow a sexually dimorphic pattern.**

One of the noticeable human lineage-specific CNVs in primates is newly named Olduvai (9), which encodes for what was originally called DUF1220,

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a protein domain of an unknown function (10). DUF1220 was originally found in a survey of genome-wide CNVs among human and great ape lineages (11). The coding sequence for this protein domain is mainly located inside the NBPF gene family on chromosome 1q21.1, and it has shown a rapid rise in the number of copies, particularly in humans (12). In humans, genomic sequences encoding DUF1220 can be subdivided into six clades (CON1–3 and HLS1–3) based on sequence similarity (13). The DUF1220 protein domain has been implicated in human brain size, neuropathology, and the number of neurons across the primate lineages (14). Furthermore, recent evidence indicates that the chromosome 1q21.1 abnormality is significantly associated with the autism phenotypes (15–17). Considering the association between the number of copies of the CON1 subtype and the volume of the gray matter (14), as well as irregularities of the gray matter and Autism Diagnostic Interview-Revised (ADI-R) score domains in individuals with ASD (18), Davis and colleagues have shown a positive correlation between the CON1 copies of DUF1220 domain and the severity of main core domains of autism, including social and communication impairments in multiplex/familial cases with autism but not simplex cases (19–21). In these studies, the investigators used a collection of predominantly verbal male subjects with a wide range of phenotypes (19–21). Given that sex/gender and genetic background may affect CNVs distribution, we conducted a study of an independent population composed of sex balanced Iranian children with simplex autism to evaluate the association between DUF1220 copy number and severity of symptoms.

METHODS

Study Population

In this study, a total of 58 well-defined individuals were recruited as described previously (22). Briefly, according to the Diagnostic and Statistical Manual of Mental Disorders criteria, fourth edition, Text Revised (DSM-IV-TR), each child with autism was diagnosed by at least two professional clinicians, a child neurologist and a specialized psychiatrist. ADI-R was also used to confirm the diagnosis of autism (23,24). Subjects with other medical or genetic disorders/syndromes were excluded. Moreover, to minimize the scope of heterogeneity among our cases with autism, we excluded families with multiple children with autism (inherited forms of autism), which usually fall in moderate-to-severe symptoms, and recruited our index patients from independent and simplex families.

In summary, we used genomic DNA samples from 24 sex-balanced children with autism, 10 apparently healthy paired siblings (eight males and two females), and 24 sex-, age-, and location-matched (*i.e.*, same geographical residence) healthy controls. The information regarding demographics, ADI-R domain scores, and the verbal status of the subjects with autism are presented in Table 1. This study was performed in line with the principles of the Declaration of Helsinki. The study had the approval of the Zanjan University of Medical Sciences (ZUMS) Research Ethics Committee (ZUMS.REC.1394.265, Date: January 05, 2016).

Table 1. Demographic information of subjects with autism

Variables	
Sex, n (%)	
Male	14 (58.3%)
Female	10 (41.6%)
Age range, months (mean)	34-123 (61.6)
Communication skill, n (male/female) *	
Verbal	15 (10/5)
Non-verbal	8 (3/5)
ADI-R Domain scores, mean	
Social interaction	35.5
Social communication	42.7
Repetitive patterns	14.1
ADI-R Total	95.9

ADI-R, Autism Diagnostic Interview-Revised.
*We missed out ADI-R information for one male with autism resulting in no detail for his communication skill.

CON1 Copy Number Measurement

TaqMan real-time quantitative polymerase chain reaction (PCR) amplification was carried out in an ABI StepOnePlus™ real-time system (Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Briefly, a 20-µl reaction mixture consists of 10-µl AmpliTaq Gold PCR Master Mix, 200 nM primers, 100 nM TaqMan probe for each of the target sequences, CON1, and the reference gene, RPP30, respectively. A master mix was prepared, vortexed, and aliquoted (18 µl) into the wells of a 0.2 µl optical-grade 96-well PCR plate (Applied Biosystems, Foster City, CA, USA). Then, 2 µl of each genomic DNA sample was added to a final volume of 20 µl. All reactions were carried out in triplicates along with no-template controls. The thermal cycle conditions were: one cycle of 95°C for 10 minutes (Taq activation), followed by 40 cycles of 95°C for 15 seconds for denaturation and 60°C for 30 seconds for annealing and extension. For absolute quantification of *CON1* copies in the sample of interest, a five-point standard curve for each synthetic calibrator was generated using a dilution factor of 1/10. The program was set to monitor the complete amplification for 40 cycles. All TaqMan PCR data were captured using the StepOnePlus™ Software version 2.3 (Applied Biosystems, Foster City, CA, USA). The sequences of the primers and probes, and synthetic DNA oligomers are presented in Table 2.

Table 2. Sequences of calibrators, primers, and probes

Target		Sequence	mer
CON1	Calibrator oligomer	5′-AAT GTG CCA TCA CTT ATT CAA ATA GCC ATG GCC CTT CTG ACT CCA ACC CGC CTC ACA AGA ACA TCA AAA TCA CAT CTG AGG AAG ACA AAG TC-3′	92
	Hybrid probe	5′-CAT GGC CCT TAT GAC TCC AAC CAG CC-3′	26
	Right primer	5′-GAC TTT GTC TTC CTC AAA TGT GAT TTT-3′	27
	Left primer	5′-AAT GTG CCA TCA CTT GTT CAA ATA G-3′	25
RPP30	Calibrator oligomer	5′-GAT TTG GAC CTG CGA GCG GGT TCT GAC CTG AAG GCT CTG CGC GGA CTT GTG GAG ACA GCC GC-3′	62
	Hybrid probe	5′-TTC TGA CCT GAA GGC TCT GCG C-3′	22
	Right primer	5′-GCG GCT GTC TCC ACA AGT-3′	18
	Left primer	5′-GAT TTG GAC CTG CGA GCG-3′	18

Synthetic calibrators for the target sequence (CON1) and reference gene (RPP30) were designed in this study.
All primers and probes were adopted from Davis et al. 2014 (21).

Data Analysis

All statistical tests were two-sided, with the *P*-values below 0.05 ($p<0.05$) considered as statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 22.0 (IBM Corp., Armonk, NY, USA). Scatter plots and bar graphs were made using GraphPad Prism, version 6.07 (GraphPad Software Inc. La Jolla, CA, USA). DUF1220 subtype *CON1* copy number among clinical groups displayed Gaussian distributions that were confirmed by using the Shapiro-Wilk tests and visual inspection of their respective histograms and plots. A one-way analysis of variance (ANOVA) and t-student test were used to compare the mean value of the CON1 copy numbers across the study groups. A multiple linear regression model was employed to test the association between the CON1 copy number and ADI-R domain scores, including social interaction, communication, and repetitive pattern as independent variables while adjusting for age and sex. Co-linearity test was used to examine potential correlations between independent variables.

RESULTS

CON1 Copy Number, Mean Analysis between the Study Groups

CON1 copy number was determined in the study groups, including subjects with autism, healthy controls, and siblings (Figure 1a). The CON1 copy numbers ranged between 9–46, 13–44, and 15–40, respectively. One-way ANOVA test showed that there were no statistically significant differences in comparisons between the autism group CON1 copy number (24.46±9.32), the healthy control group (24.42±7.90), and the paired siblings (23.30±7.19) mean values [F (55, 2)=0.076 p=0.92)] (Figure 1a). Given the profound sex bias in the prevalence of autism, we divided the autism group based on sex. The CON1 copy numbers ranged between 13–44 for male and 9–46 for female subjects. Despite this classification, there was no significant difference between the CON1 copy numbers of male (23.94±8.39) and female (24.73±8.27) subjects (p=0.451). When we compared the mean of the CON1 copy number of the verbal subjects (25.00±9.11) to that of the non-verbal subjects (24.25±10.62), again, we did not find any difference here (p=0.788) (Figure 1b).

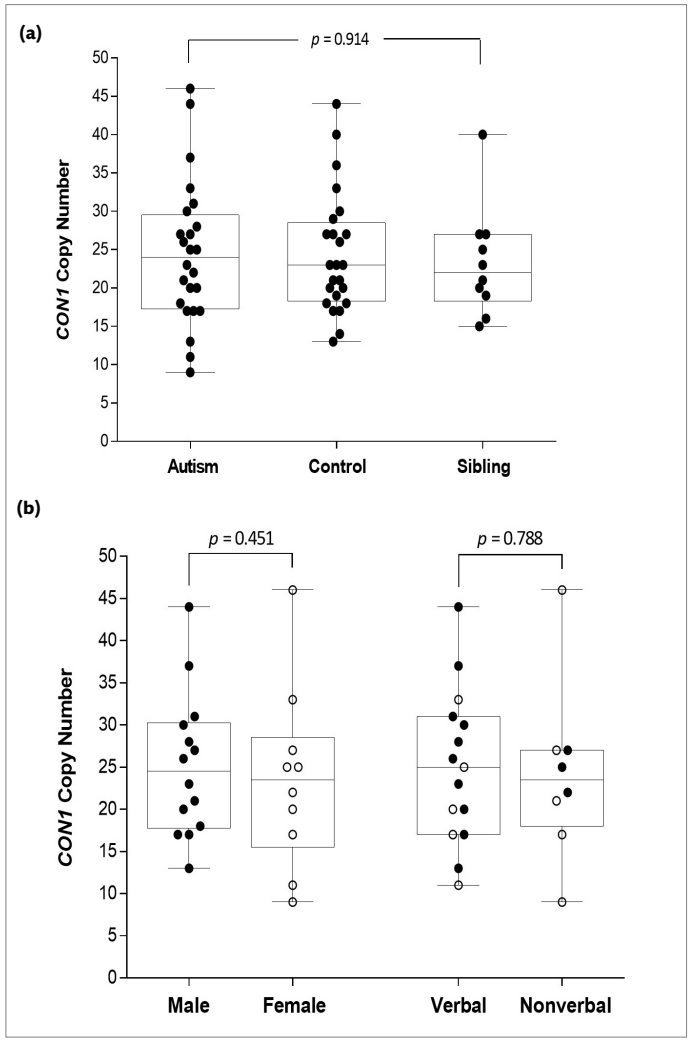

Figure 1. a, b. Frequency of the DUF1220 CON1 copy number. Comparison of the CON1 copy number in the study groups. Subjects with autism, healthy controls, and paired siblings **(a).** Patients with autism stratified based on sex (left) and communication skill/verbal status (right) **(b).** The bottom and top of each box represent the 25th and 75th percentiles, respectively. The line bisecting each box shows the mean value. Open circles in panel B indicate the female cases.

Association Analysis between CON1 Copy Number and scores of ADI-R main domains in Sex-based Classification

A multiple linear regression test was performed to find possible associations between the number of CON1 copies and each of the ADI-R domain scores of children with autism [see the supplementary Table S2 in Panahi et al. (22)]. In agreement with a recent study that was conducted by Davis et al. and included 64 subjects with simplex autism (19), our findings revealed no significant associations between ADI-R core domain scores and the number of CON1 copies in simplex cases (Table 3). However, a different pattern was observed when children with autism were classified based on sex. As seen in Figure 2, the trend of the total ADI-R scores in males was positive with increasing the number of CON1 copies (Figure 2a). In contrast, for the female subjects, this trend was somewhat reversed such that with increasing the number of CON1 copies, the ADI-R scores decreased (Figure 2b). However, these opposing trends were not statistically significant (male, p=0.11; female, p=0.69). Specifically, with each additional copy of CON1, the social interaction and communication diagnostic scores had an ascending trend in the male subjects, which would be in line with the observations of Davis et al. for the inherited form (*i.e.*, multiplex) of autism (19–21). By contrast, in the females, trends for the social interaction and communication

Table 3. Association between ADI-R core domains score and CON1 copy number

Outcome	Beta	SE	P-value
ADIR-total	0.107 point increase per copy increase of CON1	0.002	0.626
Social interaction	0.082 point increase per copy increase of CON1	0.002	0.692
Social communication	0.039 point increase per copy increase of CON1	0.006	0.856
Repetitive pattern	0.052 point increase per copy increase of CON1	0.003	0.816

ADI-R, Autism Diagnostic Interview-Revised; SE, Standard eror.
Beta estimates, standard errors and P-values are from multivariate regression.

diagnostic scores were in the opposite direction, showing a descending pattern. Also, interestingly, the repetitive pattern scores decreased in males with an additional CON1 copy number in contrast to the female subjects, who showed a positive trend (Table 4). Finally, when social scores and repetitive pattern scores in combined non-verbal subjects were regressed to CON1 copies, the results indicated that those domains were positively regulated (Figure 3a and b).

DISCUSSION

Several studies have shown that CNVs account for genetic diversity in diverse populations with different ethnical backgrounds (25–27). We conducted this study on Iranian families who had different genetic backgrounds and environments in comparison to the studies done by Davis and colleagues (19–21). Our well-defined study subjects included a well-balanced number of both sexes with non-syndromic, simplex/non-familial childhood autism who fall in the "moderate to severe" clinical

Table 4. Results from multivariate regression analyses

Sex	Outcome	Beta	SE	P-value
Male	Interaction	0.306 point increase per copy increase of CON1	0.005	0.348
	Communication	0.272 point increase per copy increase of CON1	0.009	0.376
	Repetitive pattern	-0.089 point decrease per copy increase of CON1	0.006	0.786
Female	Interaction	-0.700 point decrease per copy increase of CON1	0.001	0.074
	Communication	-0.229 point decrease per copy increase of CON1	0.008	0.570
	Repetitive pattern	0.539 point increase per copy increase of CON1	0.003	0.183

SE, Standard eror.
Beta estimates, standard errors and P-values are from multivariate regression analysis.

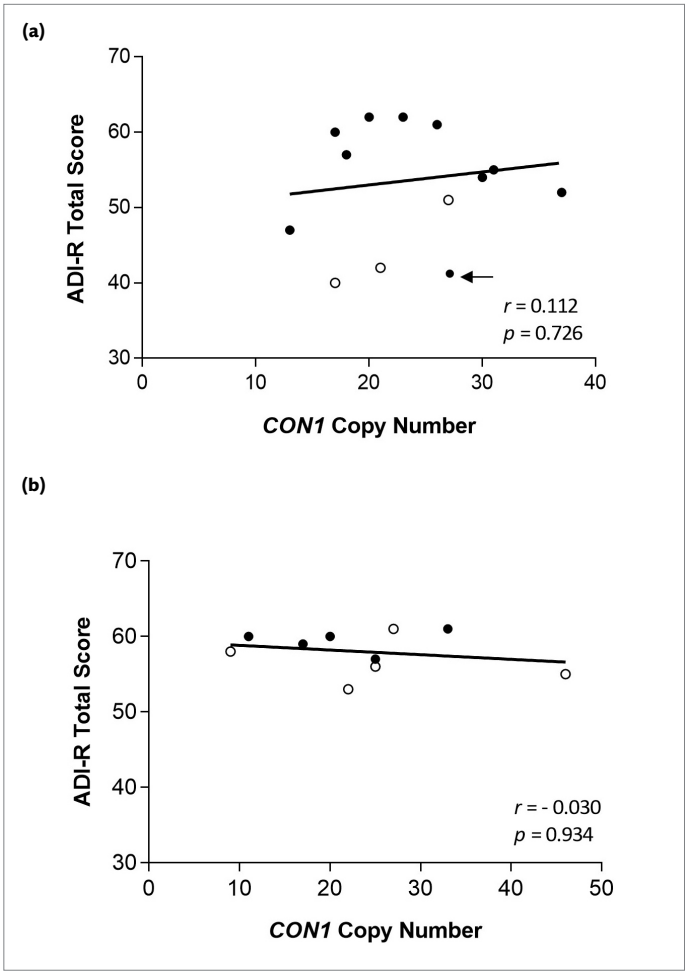

Figure 2. a, b. Association analysis between the DUF1220 CON1 copy number and total Autism Diagnostic Interview-Revised (ADI-R) scores. Correlation between CON1 copy numbers and total ADI-R scores in based on sex, male **(a)** and female **(b)** children with autism show different trends. Open circles indicate the non-verbal subjects. The arrow shows an omitted point as an outlier from the assessment.

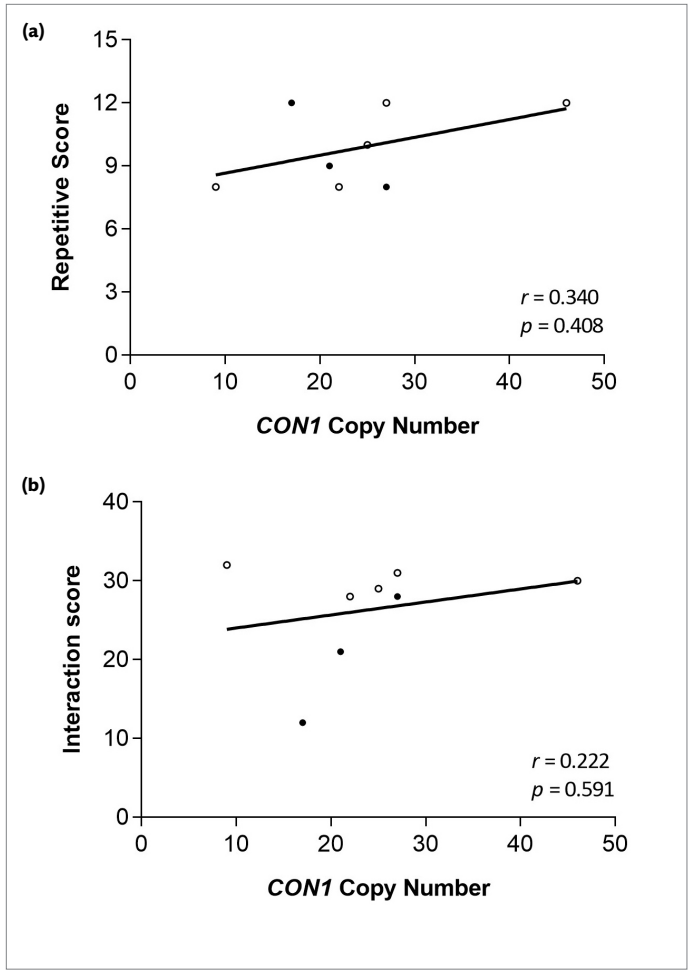

Figure 3. a, b. Association analysis between the DUF1220 CON1 copy number and Autism Diagnostic Interview-Revised (ADI-R) domain scores in non-verbal subjects. Correlation between CON1 copy number and repetitive domain scores **(a),** and interaction domain scores **(b).** Open circles indicate the female subjects.

bracket. In contrast, Davis's study population consisted of predominantly male and verbal ASD subjects, the majority of whom were recruited from multiplex families (19). Despite these differences with Davis's study in a sex-combined analysis, our results generally support their findings. However, in sex-based classification, the social interaction and communication scores for male children with autism show a positive trend with each additional CON1 copy number, similar to the observation in multiplex cases reported by Davis and colleagues. By contrast, the social interaction and communication scores for female children with autism show a negative trend, suggesting a possible protective role for CON1 copies in females with autism from simplex families (Table 3). Some studies have shown that CNV alterations may affect the expression of genes encompassing these segments (4), and ethnicity/race differences and sex can affect the number of CNVs (28). Nonetheless, our findings need to be re-examined in a larger sample size consisting of comparable numbers of both sexes.

There are a number of possible explanations for why we did not obtain statistically significant results. First, similar to Davis's study (19), there was no association between ADI-R diagnostic scores and severity in simplex patients. This result could be because multiplex cases of autism, which typically represent inherited cases, may be phenotypically and genetically distinct (29-31) from simplex cases that predominantly (52%-67%) result from *de novo* events (8,32,33). Second, our sample size may not be large enough to get statistically significant results, especially for female subjects. Notably, however, even a recent large-scale genome study found genes related to motor neurons dysfunction in simplex autism but no genes associated with the core characteristics of ASD (34).

It should also be noted that the use of diagnostic algorithms such as ADI-R could lead to various results in different studies for different reasons such as non-uniformity of ADI-R question scores in contrast to each other, different skill levels of various evaluators, variations in the accuracy of the answers provided by parents/caregivers, and the heterogeneity of autism phenotypes.

Due to the heterogeneity of autism, it seems that examining the factors affecting the severity of autism first requires a standard protocol to assess the severity of the disorder across the globe. This is because there is no comprehensive protocol available yet to assess the severity of symptoms in autism accurately, so the severity of the disease is often estimated based on clinical signs. Accordingly, finding markers such as DUF1220 CNVs to assess the severity of symptoms may be helpful. It is also necessary to consider the impact of confounding factors such as sex and various environmental factors to provide a clear picture of the manifestation of the symptom severity in autism.

Despite the emphasis on including both sexes in preclinical research, unfortunately, clinical studies historically have overlooked it so far (35,36). We recently showed that the telomere length in non-syndromic simplex childhood autism follows a sexually dimorphic pattern by including a well-balanced number of both sexes (22). In the present study, by using the same samples, the CON1 copy numbers appear to follow opposing trends in their association with the severity of symptoms in male vs. female children with autism. However, considering the statistical analysis presented (Table 3), there is no significant association between the CON1 copy numbers and the severity of symptoms in children with autism from simplex families. Another possible interpretation, would be that the DUF1220 copy number may follow a sexually dimorphic pattern regarding its association with the severity of symptoms in non-inherited cases of autism. However, this should be re-examined in a larger sample size composed of comparable numbers of both sexes.

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Ethics Committee Approval: This study was performed in line with the principles of the Declaration of Helsinki. The study had the approval of the ZUMS Research Ethics Committee (ZUMS. REC.1394.265, date: Jan. 05, 2016).

Informed Consent: All children had signed informed consent provided by their parents or caregivers.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- MP; Design- MP; Supervision- MP; Resource- Zanjan University of Medical Sciences; Materials- ME, MRE; Data Collection and/or Processing- ME, YP; Analysis and/or Interpretation- MP, YP; Literature Search- ME, YP; Writing- ME, YP; Critical Reviews- MP.

Conflict of Interest: The authors declared that there is no conflict of interest.

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