CTRP3 as a novel biomarker in the plasma of Saudi children with autism

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Background, C1q/tumor necrosis factor-related protein-3 (CTRP3) has diverse functions: anti-inflammation, metabolic regulation, and protection against endothelial dysfunction. **Methods.** The plasma level of CTRP3 in autistic patients (n=32) was compared to that in controls (n=37) using ELISA. **Results.** CTRP3 was higher (24.1% with P˂0.05) in autistic patients than in controls. No association was observed between CTRP3 and the severity of the disorder using the Childhood Autism Rating Scale (CARS). A positive correlation between CARs and the age of patients was reported. Receiver operating characteristic (ROC) analysis demonstrated a low area under the curve (AUC) for all patients (0.636). Low AUCs were also found in the case of severe patients (0.659) compared to controls, but both values were statistically significant (P≤0.05). Despite the small sample size, we are the first to find an association between CTRP3 and autism spectrum disorder (ASD).

1 **Title: CTRP3 as novel biomarker in plasma of Saudi children with Autism spectrum**

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25 **CTRP3 as novel biomarker in plasma of Saudi children with autism spectrum disorders**

Abstract

Background, C1q/tumor necrosis factor -related protein-3 (CTRP3) has diverse functions: anti-inflammatory,

metabolic regulation, and protection against endothelial dysfunction.

Methods. Plasma level of CTRP3 in autistic patients (n=32) was compared to controls (n=37) using ELISA.

Results. CTRP3 was higher (24.1% with P<0.05) in autistic patients compared to controls. No association observed

between CTRP3 and severity of the disorder using Childhood Autism Rating Scale (CARS). A positive correlation

between CARs and age of patients was reported. Receiver operating characteristics analysis (ROC) demonstrates

low area under the curve (AUC) for the total patients (0.636), it also presents low AUCs in case of severe patients

(0.659) compared to controls but both values were statistically significant (P≤0.05).

Despite the small sample size, we are the first to find association between CTRP3 and

autism spectrum disorder (ASD).

Keywords:

Autism spectrum disorder, biomarker, C1q/tumor necrosis factor (TNF)-related protein (CTRP3), Childhood Autism

Rating Scale, Receiver Operating Characteristics (ROC).

Introduction

Autism spectrum disorder (ASD) is a biologically neurodevelopmental disorder afflicting about one in every 59

children, and it is expected to increase globally. (Bjørklund et al. 2018).   The increasing prevalence of ASD made it

a high priority for scientists and health care providers, and has also attracted the public attention (Sheldrick and

Carter 2018; Xu et al. 2018). It is diagnosed   behaviorally based on a triad of symptoms, including impairment in

communication, impairment in sociability and abnormal and stereotypic behavior (Bjørklund et al. 2018). These

core symptoms can be detected before the age of three years and are lasting for the whole lifetime (Andres 2002).

Recent studies mainly focus on the mechanism and the pathogenesis of ASD. Many biomarkers which are

noninvasive quantitative measures gave precise indication to certain mechanisms can be used to give better

53   understanding of the etiological mechanisms of autism and there after its treatment. Blood is considered as a

54   potential source for the detection of many diseases because it contains huge numbers of proteins associated with the

55   physiology or pathology of diseases (Yao et al., 2021).Finding valid and predictive biomarkers for this disorder will

56   improve earlier diagnosis and intervention. Until now no specific biomarker was found to cause autism, but

57   comparing autistic patients with peers without ASD can give better understanding of the disease.

58   Many biomarkers were related to ASD and had significant role in its pathogenesis, immunological/inflammatory

59   markers are considered one of these biomarkers. Moreover, it was found that some ASD biomarkers were generated

60   from lipid abnormalities (El-Ansary and Al Dera 2016).

61   C1q/tumor necrosis factor (TNF)-related proteins (CTRPs) family a paralogue of adiponectin, was discovered.
62   There are 15 members extending

63   from CTRP1 to CTRP15, and each member is composed of four different domains, an N-terminal signal domain, a

64   short variable peptide, a collagen-like peptide, and a C-terminal globular like C1q domain (Ahima et al. 2006). Both

65   CTRPs and adiponectin are a portion of the C1q/TNF protein, which are higher in molecular weight due to the

66   presence of extra C1q domain proteins (Yi et al. 2012).

67   The CTRP family members has multiple physiological effects on metabolism, inflammation, protection against

68   endothelial dysfunction and angiogenesis.

69   CTRP3 is a novel member of this family with multiple biological functions (Peterson et al. 2010). It is detected in

70   many tissues and organs, including the heart, liver, adipocytes, cartilage, blood vessels, monocytes, fibroblasts,

71   colon, small intestine, pancreas, kidney, and brain (Schaffler and Buechler 2012).

72   CTRP3 is considered a strong proangiogenic and neuroprotective adipokine. CTRP3 attenuated secondary brain

73   injury after intracranial hemorrhage (ICH) in rats; it decreased brain edema, preserved the blood-brain barrier

74   (BBB), reduced neurological deficit and encouraged focal angiogenesis. It applies its protective role mainly via an

75   AMPK/HIF-1α/ VEGF-signaling pathway (Wang et al. 2016).   CTRP3 also exerts its protective effect during ICH

76   by inhibiting oxidative stress via PKA/NADPH signaling (Yang et al. 2017).

77   But, the relation between CTRP3 and ASD is not known yet. Whether CTRP3, which is an important member of the

78   recently discovered adipokine family, act as a promotor or inhibitor of ASD has not been studied before. Therefore,

79 the goal of this work was to measure the level of CTRP3 in autistic children and compare them with peers without

80 ASD.

Material and Methods

Study Population

83 This case-control study was conducted on 32 children with a diagnosis of ASD according to the 5th edition of the

84 diagnostic and statistical manual of mental disorders criteria (American Psychiatric Association 2013). Patients

85 recruited from the Autism Research and Treatment Centre, Department of Physiology, King Saud University,

86 Riyadh, Saudi Arabia. The autistic group composed of 32 males. Their ages ranged between 3 and 12 years (mean±

87 SD = 7.98 ± 2.59 years). Patients who were associated with neurological disease (such as palsy and tuberous

88 sclerosis), metabolic disorders (e.g., phenylketonuria, diabetes), and autoimmune disease were excluded from the

89 study, as metabolic disorders and autoimmunity may influence the results of plasma CTRP3 levels.

90 The control group formed of 37 age- and sex-matched healthy children, they were collected as previously described

91 by Mustafa and Al-Ayadhi., 2015. Their ages ranged between 3 and 12 years (mean ± SD = 7.83 ± 2.64 years). They

92 were not related to the children with ASD, and had no clinical findings indicative of immunological, diabetic,

93 chronic diseases or neuropsychiatric disorders. The control group was the healthy older brothers of the healthy

94 children who visit the Well Baby Clinic, King Khalid University Hospital, Faculty of Medicine, King Saud

95 University, Riyadh, Saudi Arabia for their regular growth assessment (Mustafa and Al-Ayadhi., 2015).

96 This study received approval from the Ethical Committee of King Khalid University Hospital (E-10-220). An

97 informed written consent was signed by the parents or the legal guardians prior to inclusion in the study.

Study Measurements:

Clinical evaluation of autistic patients:

101   Clinical evaluation depends on history taken from caregivers, clinical examination and neuropsychiatric evaluation.

102 Childhood Autism Rating Scale (CARS) was used to assess the severity of the disease (Mick 2005). This scale rates

103 the child from one to four in each of fifteen areas (relating to people; emotional response; imitation; body use; object

104 use; listening response; fear or nervousness; verbal communication; non-verbal communication; activity level; level

105   and consistency of intellectual response; adaptation to change; visual response; taste, smell and touch response and

106   general impressions)(Ozonoff, et al., 2005). According to the scale, children who have scored 30–36 have mild to

107   moderate ASD (n=9), while those with scores ranging between 37 and 60 points have a severe degree of ASD

108   (n=21) (Schopler et al., 1988).

109   **Blood sample collection.** Ten ml of blood sample was collected in anticoagulant (EDTA) tubes from all the

110   participants after overnight fasting as was previously described by Qasem et al (2016). Plasma and RBCs were

111   collected after centrifugation at 1000 rpm and stored at specific temperatures till used for analysis.

112   **Assessment of plasma CTRP3 level.** Levels of trimeric CTRP3 (<100 kDa) were measured by BioVendor

113   human CTRP3 ELISA according to manufacturer's protocol, with an intra-assay coefficient of variation of less than

114   10%. Samples with CTRP3 levels below the detection limit of the assay were assigned the lowest detectable value

115   (0.001 µg/ml). To increase accuracy, all samples were investigated as duplicate independent assays to avoid

116   inter-assay variations and to guarantee reproducibility of the obtained results (P > 0.05).

117   **Statistical analysis.** A software program was used for the statistical analysis, and results were expressed as mean

118   ± S.D. All statistical comparisons were made by independent Student's t-test, with P value < 0.05 considered

119   significant. A Wilcoxon-Mann Whitney test usually used when data were not normally distributed (Shapiro-Wilk's

120   test negative). The relationship between the CTRP3, CARs, and age was calculated using the Spearman correlations

121   test, and positive or negative correlations are listed. The receiver operating characteristics (ROC) analysis was

122   performed as an excellent statistical tool for the assessment of the effectiveness of biomarkers; it was done using the

123   same computer software. The area under the curve (AUC) was calculated to find how a plasmatic marker can

124   discriminate   between ASD patients and healthy control participants.

125

126   Results:
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128   Demographic data:

129   The demographic characteristics of children with ASD and their matched controls are shown in Table 1. Among the

130   ASD patients, 9/30 were mild-moderate, and 21/30 were severe.

131 **Levels of CTRP3 in total and subgroups of ASD patients compared to neurotypical healthy controls:**

132 The Shapiro test and boxplot showed that the CTRP3 data in children with ASD and healthy controls were not

133 normally distributed, with P values of 0.03 and 0.005, respectively (Table 2). Tables 3 and 4 together with Figure 1

134 demonstrate the significant increase in CTRP3 in the plasma of children with ASD compared to age- and sex-

135 matched controls. ASD patients recorded 24% higher CTRP3 plasma levels than healthy controls (P < 0.05), while

136 there was no significant difference between mild-moderate and severe autistic children (P < 0.127), despite the 38%

137 recorded increase, as shown in Table 4. Boxplots (Figure 1) shows data distribution in control, total ASD, mild-

138 moderate, and severe ASD patients. CTRP3 level, the standard deviation, and the box length as measure of data

139 dispersion of the ASD patients are considerably higher than control healthy participants, and of the severe compared

140 to mild-moderate patients.

141

142 **Spearman correlations between CTRP3, age, and CARS**:

143 Table 5 and Figure 2 present the Spearman correlations among CTRP3, age, and CARS. Whereas this marker did

144 not show any independent correlations with age and CARS (P < 0.092 and 0.750, respectively), both variables (age

145 and CARS) were negatively correlated (P < 0.044). The partial correlation between CTRP3 and the CARS while

146 controlling for age was not significant, with a correlation co-officiant of -0.210 (p = 0.361).

147 **Data from ROC analysis:**

148 Table 6 demonstrates the AUCs, specificity, and sensitivity of all ASD patients as well as the mild-moderate and

149 severe subgroups compared to control subjects and severe autistic patients compared to mild-to-moderate

150 participants. Based on the fact that an AUC of 0.9–1.0 shows an excellent predictive value of a biomarker, 0.8–0.9

151 means a very good marker, 0.6–0.7 means a satisfactory marker, and 0.6 means a useless marker, the AUC of all

152 patients compared to controls (AUC of 0.636) and that of severe autistic patients compared to controls (AUC of

153 0.659) are low but within the satisfactory value (AUC of 0.6–0.7).

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155

156   Discussion;

157

158   C1q TNF related protein 3 (CTRP3) is a relatively novel hormonal factor primarily derived from adipose tissue and

159   plays a role in early childhood development (Kown et al. 2018). In the present study, the remarkable higher level of

160   CTRP3 in plasma of autistic patients compared to healthy and gender matching controls can be easily related to the

161   disruption of BBB as phenotypic feature in ASD. As a TNF-related protein the increase of CTRP3 is in good

162   agreement with multiple studies in which TNF-α was significantly increased in blood and positively correlated with

163   severity of ASD (Xie et al. 2017).

164   In addition, Jyonouchi and collaborators (2001) found that TNF-α was elevated in the autistic subjects, and majority

165   of those autistic children exhibited excessive or poorly regulated innate immune responses. Moreover, Chez et al.

166   (2007) found increased TNF-α in the cerebrospinal fluid of autistic children, and it was also significantly increased

167   in the brains of autistic subjects (Li et al. 2009). Although TNF-α was decreased in Saudi autistic patients compared

168   to peers without ASD, this was attributed to the early increase in plasma followed by effluxto the brain through the

169   BBB (El-Ansary and Al-Ayadhi 2014). It is well known that, binding with ligand, TNF-α can activate NF-κB,

170   MAPK, and the apoptosis signaling pathway (Perry et al. 2001). Based on this the significant increase of CTRP3 in

171   the present study can be easily related to the impaired NF-κB, MAPK, and activated apoptosis signaling pathway

172   repeatedly recorded in autistics compared to peers without ASD (Young et al. 2011, 2012; Naik et al. 2011). A study

173   done by Qasem et al. (2018) reported a significant increase of NF-κB in plasma of Saudi patients with autism. Xu et

174   al. (2015) suggest that TNF-α might affect the progress of ASD through another pathway, such as the MAPK/JNK

175   pathway which can be related to the increase of related CTRP3 reported in the present study.   Gomez-Fernandez et

176   al (2018) showed that there is no significant difference in the level of the expression of relevant plasma cytokines,

177   cell adhesion molecules or growth factors in children with ASD compared with peers without ASD.

178   CTRP 3 is definitely induced during late stage of adipocyte differentiation and triggers secretion of adiponectin with

179   certain regulatory metabolic functions (Yi et al. 2012). Current data proves that CTRP3 is functionally the most

180   similar homolog of adiponectin (Schaffler and Buechler 2012) and intravenously injected CTRP3 can cross the BBB

181   and increase the adiponectin levels in the cerebrospinal fluid (Wang and Scherer 2016). Recently, it was found that

182   total CTRP3 concentrations were significantly positively correlated with total cholesterol and HDL cholesterol in

183   children aged 7–10 years (Alamian et al., 2020). Based on this finding, the reported increased level of CTRP3 in

184   autistic patients (Table 3 and 4) could help to support the association between CTRP3 and metabolic diseases and

185   could find support in the recent multidimensional precision medicine approach, which identifies subgroups of

186   individuals with ASD characterized by dyslipidemia (Luo et al., 2020; Josiane da et al., 2021).

187   Protein kinases are essential in G-protein-coupled, receptor-mediated signal transduction and are involved in

188   neuronal functions, gene expression, memory, and cell differentiation. The cAMP–PKA pathway is one of the most

189   common signaling pathway (Castro et al. 2013). The activity and expression of protein kinase A (PKA), a cyclic

190   AMP–dependent protein kinase, in different areas of postmortem brain of individuals with ASD demonstrated

191   significantly lower level of PKA compared to healthy control subjects. PKA signaling can counteract superoxide

192   anion accumulation and prevent SOD and catalase inhibition induced by oxidative stress in cultured astrocytes

193   (Douiri et al. 2016). Based on the fact that Saudi autistic children are under H_2O_2 oxidative stress due to

194   overexpression of SOD with slightly lower catalase, cAMP-PKA can be given special attention (Al-Gadani et al.

195   2009). PKA signaling mediates CTRP 3's anti-oxidative effects during brain injury have yet to be understood. PKA

196   is involved in CTRP 3 -mediated suppression of ROS in endothelial cells (Goldstein et al. 2009), Yang et al. (2017)

197   confirmed a role for PKA in the protective effects of CTRP 3's against brain injury. It is commonly known that the

198   BBB plays an essential role in the protection of the brain by limiting the influx of circulating harmful solutes,

199   macromolecules, and cells from the blood into the brain. However, numerous studies have revealed that dysfunction

200   of the BBB is associated with the pathogenesis of neurological disorders, including ASD, suggesting that some

201   ASD-related proteins might be secreted from the brain into the blood as potential biomarkers. (Theoharides and

202   Doyle, 2008; Theoharides et al., 2008; Theoharides and Zhang, 2011; Fiorentino et al., 2016).

203   Based on this the remarkable increase of plasma CTRP3 in the present study can be related to BBB disruption and to

204   brain oxidative stress as confirmed etiological mechanism in ASD. With a disrupted BBB as autistic feature, the

205   recorded increase of CTRP3 could be concomitant with remarkable decrease in the brain due to efflux from brain to

206   blood.   A study of Rai-Bhogal et al. (2018) confirmed the inhibition of PKA signaling as a pre-requisite of CTRP3

207   protective effects with prostaglandins as pro-inflammatory lipid mediator. Prostaglandins were among the elevated

208   lipid mediators previously reported by El-Ansary and Al-Ayadhi (2012) in plasma of Saudi autistic patients

209   compared to peers without ASD . In a most recent study done by Qasem et al. (2018) PGE2 and mPGES-1 were

210   positively correlated with NF-κB as proinflammatory marker and associated with the dysfunction in sensory

211   processing.

212   Table 6 demonstrates the AUCs, specificity, and sensitivity of all ASD patients compared to control subjects and

213   severe autistic patients compared to mild-to-moderate participants. The recorded AUC of all patients compared to

214   controls (AUC of 0.636) and that of severe autistic patients compared to controls (AUC of 0.659) are low but within

215   the satisfactory value known for ROC AUC (AUC of 0.6–0.7). This could help to accept CTRP3 as a novel

216   predictive biomarker of ASD.

217   Conclusion:
218

219   CTRP3 is a novel biomarker never measured in plasma of patients with ASD, CTRP3 was higher in autistic patients

220   compared to controls. Therefore, can have a role in the early diagnosis of this disorder.

221   The data of the current study are hindered by the relatively small sample number of participants, and will, therefore,

222   need to be replicated by larger size of both individuals with ASD and controls. Also, all the recruited subjects in this

223   study were males, so the conclusions cannot be anticipated to be present in females with ASD. The inclusion of

224   both sexes in our future studies may help to clarify the sex differences in this disorder.

225   Acknowledgements:

226   This project was funded by the National Plan for Science Technology and Innovation (MAARIFAH), King

227   Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award number: 08-MED 510–02. The

228   authors want to extend their thanks to the Autism Research and Treatment Center, Riyadh, Saudi Arabia for

229   providing blood samples from children with autism and the control group. Special thanks to all the participants and

230   their families for taking part in this study.

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233   **Compliance with Ethical Standards**

234   **Conflict of interest**

235   The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

236   **Ethical approval**

237   All procedures performed were in accordance with the ethical standards of the institutional and/or national research

238   committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Demographic of children with autism and healthy control

2 **Table 1 :** *Demographic of children with autism and healthy control*

		Children with autism (n= 32)	Control group (n=37)
Age (in years)	Range	3-12	3-12
	Mean ± SD	7.98 ± 2.59	7.83 ± 2.64
Sex	Male	32 males	37 males
CARS scores	Mild to moderate (30–36)	9	-------
	Severe (37–60)	21	-------

Table_2_CTRP3

2 **Table 2: Test of Normality using Shapiro Test.**

Parameters	Groups	N	P value
CTRP3 (ug/ml)	Control	37	0.005
	Patients	32	0.033
CTRP3 (ug/ ml)	Mild to Moderate	8	0.041
	Severe	20	0.153
Age (Years)	Mild to Moderate	5	0.037
	Severe	15	0.269

5 If P value less than or equal to 0.05 then the data is not normal distributed and If P value more
6 than 0.05 then the data is normal distributed
7

Mean ± S.D. of CTRP3 in plasma of total autistic patients compared to control subjects.

3   Table 3: *Mean ± S.D. of CTRP3 in plasma of total autistic patients compared to*

4   *control subjects.*

Groups	N	Min.	Max.	Mean ± S.D.	Percent Change	P value
Control (ug/ml)	37	0.12	0.68	0.33 ± 0.14	100.00	0.050
Patients (ug/ml)	32	0.13	0.99	0.41 ± 0.18	124.71	

7   *Comparing between groups using Mann-Whitney test

Mean ± S.D. of CTRP3 in plasma of Mild to Moderate and Severe autistic patients.

2
3   Table 4: *Mean ± S.D. of CTRP3 (*ug/ml) *in plasma of Mild to Moderate and Severe*
4   *autistic patients.*

Groups	N	Min.	Max.	Mean ± S.D.	Percent Change	P value
Mild to Moderate	8	0.20	0.58	0.31 ± 0.12	100.00	0.127
Severe	20	0.13	0.99	0.43 ± 0.20	138.16	

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8

Spearman correlations between CTRP3, CARs,

1   Table 5: *Spearman correlations between CTRP3, CARs,*

Parameters	R (Spearman Correlation)	P value	
CTRP3 with Age	-0.368	0.092	N[b]
CTRP3 with CARS	0.061	0.750	P[a]
Age with CARS	-0.433*	0.044	N[b]

4   • * Correlation is significant at the 0.05 level.
5   • [a] Positive Correlation.
6   • [b] Negative Correlation.

ROC-Curve of CTRP3 in all patients Group according to Control Group

1   **Table 6: *ROC-Curve of CTRP3 in all patients Group according to Control***
2   ***Group***

CTRP3 ROC	AUC	Cut-off value	Sensitivity %	Specificity %	P value	95% CI
All patients according to Control	0.636	0.341	56.2 %	70.3 %	0.050	0.503 - 0.769
Mild to Moderate according to Control	0.532	0.315	75.0 %	43.2 %	0.778	0.326 - 0.738
Severe according to Control	0.659	0.341	65.0 %	70.3 %	0.049	0.504 - 0.814
Severe according to Mild to Moderate	0.688	0.372	60.0 %	87.5 %	0.127	0.479 - 0.896

5

Figure 1

boxplot to show the data distribution of CTRP3 in the control and patients' groups (a), and data distribution of CTRP3 in mild to moderate and sever autistic groups (b).
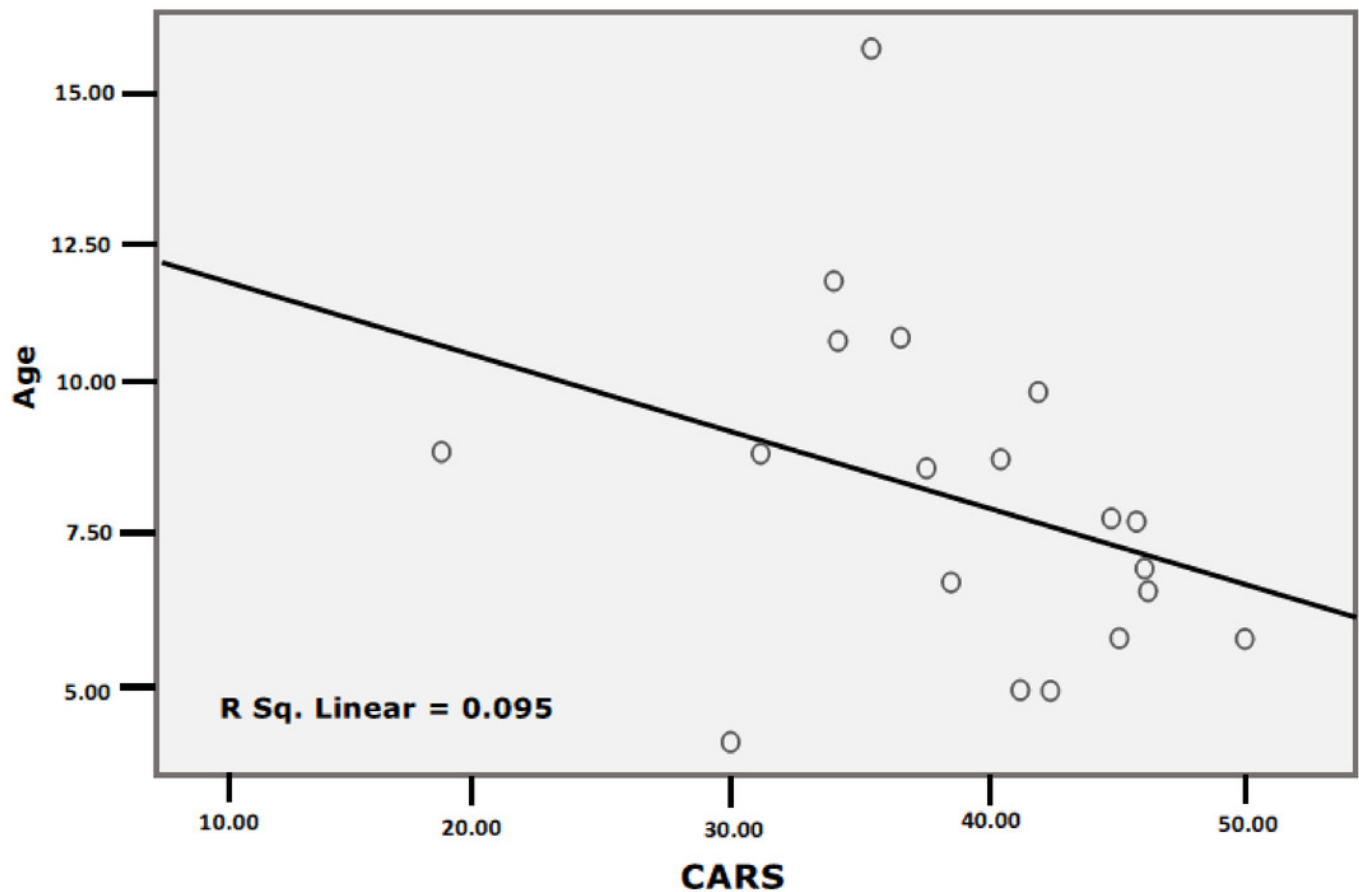
Figure 2

Correlation between CARS and Age with best fit line curve (negative correlation)