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Adiposity and Low-Grade Systemic Inflammation Modulate Matrix Metalloproteinase-9 Levels in Greek Children with Sleep Apnea

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10 Matrix Metalloproteinase-9 Levels
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12 in Greek Children with Sleep Apnea
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Abstract

Background: Matrix metalloproteinase-9 (MMP-9) plasma levels correlate with C-reactive protein (CRP) concentrations and they are both increased in adults with obstructive sleep apnea (OSA). No studies have evaluated MMP-9 levels in children with sleep apnea and CRP is not consistently elevated in pediatric OSA. The aim of this investigation was to evaluate the association of severity of OSA, adiposity and CRP with MMP-9 plasma levels in Greek children.

Methods: Consecutive children with snoring who underwent polysomnography and were found to have OSA (obstructive apnea-hypopnea index-OAHI ≥ 1 episode/hour) were recruited. Subjects without OSA (OAHI < 1 episode/hour) were included for comparison. Morning plasma MMP-9 and CRP were measured.

Results: Twenty nine children with moderate-to-severe OSA (age 5.4 ± 1.5 years; OAHI 13.9 ± 13.0 episodes/hour), 55 participants with mild OSA (6.4 ± 2.6 years; OAHI 2.4 ± 1.1 episodes/hour) and 22 subjects without OSA (6.8 ± 2.6 years; OAHI 0.6 ± 0.2 episodes/hour) were studied. Children with moderate-to-severe OSA were similar to those with mild OSA or without OSA regarding ln-transformed MMP-9 values (5.87 ± 0.60 vs. 5.84 ± 0.55 vs. 5.80 ± 0.46 ; $p > 0.05$) and CRP concentrations (0.22 ± 0.29 mg/dL vs. 0.21 ± 0.36 vs. 0.13 ± 0.16 mg/dL; $p > 0.05$). In multiple linear regression, body mass index ($p = 0.027$) and CRP levels ($p = 0.008$), but not OAHI or SpO₂ nadir ($p > 0.05$), were significantly related to MMP-9 values.

Conclusions: Adiposity and systemic inflammation unrelated to OSA severity, modulate MMP-9 levels in Greek children.

Introduction

Obstructive sleep-disordered breathing (SDB) with its associated nocturnal intermittent hypoxemia is considered an oxidative stress disorder characterized by release of inflammatory mediators, increased leukocyte adherence to endothelial cells, reduced nitric oxide availability and ultimately endothelial dysfunction and vascular injury.¹⁻³ Systemic inflammation and monocyte migration into the vascular wall have central roles in the pathogenesis of atherosclerosis and can potentially explain the epidemiologic association between obstructive sleep apnea and risk of cardiovascular events.⁴⁻⁶

Matrix metalloproteinase-9 (MMP-9) contributes to degradation of the extracellular matrix and to vascular remodelling and its plasma levels correlate with indices of systemic inflammation like C-reactive protein (CRP).^{7,8} Studies in adult sleep apneics have associated CRP and MMP-9 serum levels with severity of SDB and with the degree of adiposity.⁹⁻¹² In addition, monocytes isolated from adults with severe sleep apnea have higher production of MMP-9 than monocytes from control subjects.¹³ Treatment of sleep apnea with nasal continuous positive airway pressure diminishes serum levels of MMP-9 and its production from monocytes.^{11,13}

There are no published studies assessing MMP-9 plasma concentrations in children with obstructive SDB. It is expected that increased levels of CRP will be accompanied by elevated concentrations of MMP-9.⁸ However, the strength of the association between severity of intermittent upper airway obstruction during sleep and CRP in childhood may vary according to the ethnic population studied.¹⁴⁻¹⁹ It is known that Greek and Australian children with SDB have lower serum CRP concentrations than United States (US) children with SDB of similar severity.^{14,17,19,20} Thus, the aim of this investigation was to assess whether severity of

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2
3 intermittent upper airway obstruction during sleep, morning plasma concentration of
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5 CRP and degree of adiposity are significantly related to morning plasma MMP-9
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7 levels in Greek children with OSA. It was hypothesized that children with OSA have
8
9 increased MMP-9 levels.
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12 Patients and Methods

13 **Participants and clinical evaluation**

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17
18 The study protocol was approved by the Larissa University Hospital Ethics
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20 Committee and parents of participants provided informed consent. Consecutive
21
22 children with history of snoring and adenotonsillar hypertrophy, who underwent
23
24 polysomnography and were found to have obstructive sleep apnea (OSA) were
25
26 recruited in the study prior to adenotonsillectomy. OSA was defined as an obstructive
27
28 apnea-hypopnea index (OAHI) ≥ 1 episode/hour. Subjects with hypertrophic tonsils
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30 and without snoring, who underwent polysomnography to rule out OSA and who had
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32 OAHI < 1 episode/hour were included in the study for comparison (group without
33
34 OSA). Exclusion criteria for participation in the study were: i) symptoms or signs of
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36 acute or chronic inflammation (e.g. recurrent tonsillitis); ii) history of cardiovascular,
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38 neuromuscular or genetic disorders; and iii) use of sympathomimetics, corticosteroids
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40 or leukotriene modifiers.
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47 Study participants underwent a detailed physical examination with
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49 measurement of weight and standing height. Body mass index (BMI) and BMI z-
50
51 score were calculated. Obesity was defined as BMI z-score > 1.645 .²¹
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53 **Polysomnography**

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56 Participants underwent overnight polysomnography in the Sleep Disorders
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58 Laboratory of Larissa University Hospital. The following parameters were recorded:
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60 electroencephalogram (C3/A2, C4/A1, O1/A2); right and left oculogram; submental

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3 and tibial electromyogram; body position; electrocardiogram; thoracic and abdominal
4 wall motion; oronasal airflow (3-pronged thermistor); and oxygen saturation of
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6 hemoglobin.
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10 Arousals were scored according to recommendations provided in the
11 American Academy of Sleep Medicine Manual.²² Obstructive apnea was defined as
12 a >90% fall in the airflow signal amplitude compared to the pre-event baseline, lasting
13 for at least 2 missed breaths and in the presence of continued or increased inspiratory
14 effort.²² An apnea was considered as mixed if part of the event was associated with
15 absent inspiratory effort and the remaining part with the presence of respiratory
16 movements. Hypopnea was scored when there was a decrease in the airflow signal
17 amplitude of at least 50% compared to the pre-event baseline, that lasted for at least 2
18 missed breaths and which was associated with an arousal or $\geq 3\%$ desaturation.²²
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20 OAHl was equal to the average number of obstructive and mixed apneas and
21 hypopneas per hour of sleep.
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36 **Measurement of plasma MMP-9 and CRP**

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38 Venipuncture was completed in the morning after polysomnography. The
39 blood sample was collected into a tube containing heparin and was immediately
40 centrifuged. Part of the supernatant (platelet-free plasma) was aliquoted and frozen at
41 -70° C for later measurement of MMP-9 concentration, and the remaining sample was
42 processed for determination of CRP level.
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51 A high sensitivity immunonephelometric method with lowest detection limit
52 of 0.0175 mg/dL (N High Sensitivity CRP; Dade Behring, Marburg, Germany) was
53 used for quantification of CRP levels. MMP-9 concentration was measured with a
54 commercially available solid phase ELISA (Quantikine MMP-9 Immunoassay; R&D
55 Systems Inc., Minneapolis, MN). The assay has a lowest detection limit of 0.156
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3 ng/mL, intra-assay coefficient of variation 1.9%-2.9% and inter-assay coefficient of
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5 variation 6.9%-7.9%.
6
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8 **Data analysis**

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10 Morning plasma MMP-9 level was the primary outcome measure of the
11
12 current investigation. MMP-9 concentrations were log-transformed (natural
13
14 logarithm) to approach a normal distribution. Three study groups were formed: i)
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16 subjects without OSA (OAHl < 1 episode/hour); ii) children with mild OSA (OAHl =
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18 1-5 episodes/hour); and iii) subjects with moderate-to-severe OSA (OAHl > 5
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20 episodes/hour).
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25 The 3 study groups were compared in terms of subjects' characteristics,
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27 polysomnography indices, CRP and MMP-9 plasma levels. One-way analysis of
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29 variance (ANOVA) followed by post-hoc tests (Bonferroni's test) was carried out for
30
31 comparisons of study groups regarding continuous variables and χ^2 test (Yate's
32
33 correction) for comparisons regarding categorical characteristics. Analysis of
34
35 covariance was also used to compare the 3 study groups in terms of ln-transformed
36
37 MMP-9 taking under consideration the fact that these groups differed significantly in
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39 BMI z-score.
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44 Univariate Pearson's correlation was applied to evaluate the association of ln-
45
46 transformed MMP-9 with BMI z-score, polysomnography indices, and CRP.
47

48 Multiple linear regression analysis was completed to assess whether age, gender, BMI
49
50 z-score, and OAHl or SpO₂ nadir were related to ln-transformed MMP-9
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52 concentrations. Multivariable analysis was repeated after including CRP as an
53
54 additional independent variable in the regression analysis model.
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57 **Results**

58 **Subjects' characteristics and polysomnography findings**

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3 Eighty four children with OSA and 22 subjects without OSA were recruited.
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6 The 3 study groups did not differ in terms of age and gender. Ninety eight (92.5%) of
7
8 all children were younger than 10 years old. Participants with moderate-to-severe
9
10 OSA had significantly higher BMI z-score than participants without OSA (Table 1).
11
12 Polysomnography findings for each study group are summarized in Table 1.
13
14

15 **Morning concentrations of MMP-9 and CRP**

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17 Children with moderate-to-severe OSA, mild OSA or without OSA were
18
19 similar regarding MMP-9 and CRP plasma concentrations (Table 2 and Figure 1).
20
21 Analysis of covariance confirmed that the 3 study groups did not differ in ln MMP-9
22
23 values even after adjustment for BMI z-score ($p=0.833$). Adjusted mean (\pm standard
24
25 deviation) ln-transformed MMP-9 values for subjects with moderate-to-severe OSA,
26
27 mild OSA or without OSA were: 5.85 ± 0.54 , 5.81 ± 0.54 or 5.90 ± 0.58 , respectively.
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32 There was no correlation of ln-transformed MMP-9 levels with OAHl
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34 ($r= -0.03$; $p=0.779$), respiratory arousal index ($r= -0.07$; $p=0.456$), oxygen
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36 desaturation of hemoglobin index ($r= -0.04$; $p=0.703$) or SpO₂ nadir ($r=0.03$; $p=0.733$).
37
38 In contrast, ln-transformed MMP-9 levels were significantly associated with BMI z-
39
40 score ($r=0.21$; $p=0.032$) and CRP ($r=0.28$; $p=0.004$). In multiple regression analysis,
41
42 BMI z-score and CRP level-but not severity of SDB-were related to ln-transformed
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44 morning MMP-9 concentrations (Table 3).
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51 **Discussion**

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53 In the present study it has been demonstrated that low-grade systemic
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55 inflammation, as reflected by CRP, and degree of adiposity affect MMP-9 plasma
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57 levels in Greek children with OSA, whereas these levels are not modulated by
58
59 severity of OSA. This finding is in contrast to the reported association of MMP-9
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3 with severity of OSA in adults but in line with the previously described low CRP
4 serum levels in Greek children with sleep apnea.^{11,13,17,18} The absence of a
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7 relationship between MMP-9 and polysomnography indices in Greek children
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9 supports further the concept that different ethnic pediatric populations with OSA
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11 undergo activation of diverse inflammatory and oxidative stress pathways.
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15 Matrix metalloproteinases (MMPs) are a family of zinc-dependent
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17 endopeptidases that degrade extracellular matrix proteins.²³ MMPs are excreted as
18
19 zymogens that are activated by proteinases and their action is modified by tissue
20
21 inhibitors (TIMPs).^{24,25} They are involved in the inflammatory processes related to
22
23 atherosclerosis and plasma concentration of MMP-9 is a predictor of cardiovascular
24
25 mortality in patients with coronary disease.^{26,27} More specifically, MMPs promote
26
27 degradation of the vascular basement membrane allowing monocyte infiltration and
28
29 smooth muscle cell migration into the vascular wall, ultimately resulting in vascular
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31 remodeling and atheromatic plaque formation.^{7,28,29} Furthermore, MMP-9
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33 expression in the myocardium increases in parallel with the growing left ventricle
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35 myocardial mass in patients with ventricular pressure overload.³⁰
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42 Three studies in adults with OSA have identified increased serum MMP-9
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44 concentrations or enhanced MMP-9 production by monocytes.¹¹⁻¹³ Tazaki et al
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46 reported that MMP-9 serum levels and enzyme activity measured by gelatin
47
48 zymography were higher in men with moderate-to-severe OSA than in patients with
49
50 mild OSA or in obese controls.¹¹ MMP-9 levels and activity were positively
51
52 correlated with BMI and serum concentrations of interleukin-6 (IL-6) or tumor
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54 necrosis factor-alpha (TNF-alpha) and these levels decreased after treatment with
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56 nasal continuous positive airway pressure.
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3 A variety of cells including monocytes, macrophages, neutrophils and
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6 endothelial cells release pro-MMP-9 after stimulation by inflammatory cytokines like
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8 IL-6 and TNF-alpha.^{7,31} Hence, it is possible that hypoxemia-triggered production of
9
10 IL-6 and TNF-alpha in sleep apneics promotes synthesis of MMP-9.^{7,31-33} Since IL-6
11
12 also regulates the synthesis of CRP, it is not surprising that MMP-9 and CRP are
13
14 significantly correlated with each other in male subjects with OSA and their levels
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16 increase with increasing severity of sleep apnea.^{12,34} Augmented production of TNF-
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18 alpha and MMP-9 from peripheral blood monocytes in severe OSA decreases
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20 following treatment with nasal continuous positive airway pressure.¹³
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25 Similar to findings in adults,^{11,12} in the current report, CRP and the degree of
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27 adiposity were significantly associated with MMP-9 plasma concentrations. In vitro
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29 experiments involving peritoneal macrophages from rats have revealed that CRP
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31 enhances gene expression of MMP-9.³⁵ Moreover, in healthy obese and lean
32
33 children and adolescents MMP-9 levels increase linearly with increasing BMI.³⁶ A
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35 two-week high fiber and low-fat diet along with daily exercise have a lowering effect
36
37 on MMP-9 serum concentrations in overweight children.³⁷
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42 Although blood pressure elevation and changes in cardiac structure and
43
44 function are the main effects of pediatric OSA on the cardiovascular system,³⁸⁻⁴⁰ it is
45
46 conceivable that sleep apnea is also associated with changes in collagen turnover of
47
48 the vascular wall as it has been postulated for obese children and adolescents with
49
50 elevated MMP-9 concentrations.^{36,37} Nevertheless, as opposed to results from
51
52 investigations in adults, MMP-9 levels in this study were not affected by severity of
53
54 OSA, despite the presence of a positive correlation between plasma MMP-9 and CRP
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56 concentrations.
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3 A number of reasons could explain the discrepant results of the current study
4 compared to findings of previous reports on MMP-9 levels in adult sleep apneics.
5
6 First, adults with sleep apnea have more severe intermittent hypoxemia in comparison
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8 to children with SDB and this difference may be responsible for the higher prevalence
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10 of systemic inflammation in the former than in the latter. Second, OSA is usually
11
12 present for more years and hence its complications are more likely frequent in adults
13
14 than in young children. Third, there is emerging evidence for two variants or types of
15
16 pediatric OSA.⁴¹ There appears to be a phenotype in older and obese children and
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18 adolescents resembling sleep apnea in adults, with low-grade systemic inflammatory
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20 response, insulin resistance, dyslipidemia and inadequate improvement after
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22 adenotonsillectomy. The majority of participants of the present study were younger
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24 than 10 years old and probably belonged to the classical sleep apnea phenotype with
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26 adenotonsillar hypertrophy, less intense systemic inflammatory response and apparent
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28 improvement after adenotonsillectomy.
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36 In addition, different inflammatory and oxidative stress pathways may be
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38 activated among populations with OSA and different genetic background or
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40 environmental exposures. US children with obstructive SDB have increased
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42 concentrations of circulating vascular endothelial growth factor (VEGF), IL-6 and
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44 CRP, whereas VEGF and CRP appear unaffected by severity of OSA in Greek
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46 children.⁴²⁻⁴⁴ In contrast, excretion of uric acid, an important antioxidant and free
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48 radical scavenger, is accentuated in Greek children with SDB but it is not modulated
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50 by severity of OSA in US children.⁴⁵ Finally, urine excretion of cysteinyl
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52 leukotrienes is related to severity of SDB in Greek children.⁴⁶ Thus, genetic
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54 variability and environmental factors may be responsible for the differential oxidative
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3 stress and inflammatory responses to intermittent upper airway obstruction during
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5 sleep.⁴⁷
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8 One potential limitation of the current study is that use of nasal thermistry to
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10 monitor airflow without measurement of nasal pressure may have underestimated the
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12 frequency of hypopneas. Also, the 3 study groups were not matched in terms of BMI
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14 z-score. However, analysis of covariance confirmed that children with moderate-to-
15
16 severe OSA, mild OSA or without OSA had similar MMP-9 values even after
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18 adjustment for BMI z-score. Finally, only plasma MMP-9 levels but not activity of
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20 the enzyme were measured. While the three groups of study participants did not
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22 differ in terms of MMP-9 concentrations, it is plausible that they were different
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24 regarding MMP-9 activity due to variable levels of TIMPs.
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29 The present study represents the first attempt in the pediatric literature to
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31 assess the potential association between sleep apnea and MMP-9 levels. In the study
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33 design, the sample size has not been calculated since there is no prior estimate of the
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35 variability and the clinically significant difference of MMP-9 values (primary
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37 outcome measure) in children with and without OSA. In such novel cohort studies,
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39 the number of participants is usually determined by patient availability and resources.
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43 In conclusion, MMP-9 plasma levels in Greek children with sleep apnea are
44
45 affected by adiposity and by low-grade systemic inflammation unrelated to OSA.
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47 Severity of intermittent upper airway obstruction during sleep does not appear to
48
49 influence MMP-9 concentrations in the studied pediatric population.
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Figure Legends

Figure 1. Boxplots of ln-transformed matrix metalloproteinase-9 (MMP-9) plasma levels in: i) children without obstructive sleep apnea (OSA) (n = 22); ii) subjects with mild OSA (n = 55); and iii) children with moderate-to-severe OSA (n = 29). The three groups did not differ in MMP-9 levels (p=0.736). Horizontal bars represent median; whiskers represent highest and lowest values.

For Peer Review

Tables

Table 1. Summary statistics and significance of comparisons regarding subjects' characteristics. Continuous variables are presented as mean \pm standard deviation.

| | Subjects without OSA | Subjects with mild OSA | Subjects with moderate-to- severe OSA |
|---|-------------------------|---------------------------|---|
| | n = 22 | n = 55 | n = 29 |
| Age, years | 6.8 \pm 2.6 | 6.4 \pm 2.6 | 5.4 \pm 1.5 |
| Gender, female (%) | 11 (50) | 20 (36.4) | 9 (31.0) |
| BMI z score * | -0.1 \pm 1.5 | 1.3 \pm 1.1 | 1.3 \pm 1.5 |
| Obese (%) | 4 (18.2) | 25 (45.5) | 11 (37.9) |
| OAH1 [†] , episodes/hour | 0.6 \pm 0.2 | 2.4 \pm 1.1 | 13.9 \pm 13.0 |
| Respiratory arousal index [†] , episodes/hour | 0.3 \pm 0.2 | 1.4 \pm 1.0 | 4.8 \pm 3.5 |
| Oxygen desaturation of hemoglobin (\geq 3%) index [†] , episodes/hour | 0.9 \pm 0.6 | 3.5 \pm 2.2 | 15.6 \pm 14.1 |
| SpO ₂ nadir [†] , % | 91.3 \pm 3.6 | 89.5 \pm 2.6 | 79.9 \pm 16.2 |

* p=0.001 for the comparisons of moderate-to-severe OSA group or mild OSA group vs. the group without OSA

[†] p<0.001 for the comparisons of moderate-to-severe OSA vs. mild OSA or vs. no OSA groups

Abbreviations

| | |
|------------------------|---|
| BMI | Body mass index |
| OAH1 | Obstructive apnea-hypopnea index |
| OSA | Obstructive sleep apnea |
| SpO₂ | Oxygen saturation of hemoglobin by pulse oximetry |

Table 2. Summary statistics and significance of comparisons (one-way analysis of variance) regarding plasma levels of MMP-9 or CRP and ln-transformed MMP-9 values. Variables are presented as mean \pm standard deviation.

| | Subjects without OSA | Subjects with mild OSA | Subjects with moderate-to- severe OSA | p value |
|-----------------|-------------------------|---------------------------|---|------------|
| | n = 22 | n = 55 | n = 29 | |
| MMP-9, ng/mL | 365.2 \pm 164.3 | 400.3 \pm 252.7 | 415.7 \pm 231.6 | 0.736 |
| lnMMP-9 | 5.80 \pm 0.46 | 5.84 \pm 0.55 | 5.87 \pm 0.60 | 0.901 |
| CRP, mg/dL | 0.13 \pm 0.16 | 0.21 \pm 0.36 | 0.22 \pm 0.29 | 0.566 |

Abbreviations

| | |
|--------------|----------------------------|
| CRP | C-reactive protein |
| MMP-9 | Matrix metalloproteinase-9 |
| OSA | Obstructive sleep apnea |

Table 3. Multiple linear regression analysis models assessing the associations of morning ln-transformed MMP-9 plasma levels with indices of severity of sleep-disordered breathing, CRP, and BMI z-score.

| Model 1 Dependent variable: ln MMP-9 p=0.157 | | | Model 2 Dependent variable: ln MMP-9 p=0.357 | | |
|---|---------------------------|-----------------|---|---------------------------|-----------------|
| Parameters | β | p values | Parameters | β | p values |
| Age | 0.019 | 0.922 | Age | 0.010 | 0.920 |
| Gender | 0.119 | 0.236 | Gender | 0.127 | 0.216 |
| BMI z-score | 0.256 | 0.014 | BMI z-score | 0.191 | 0.063 |
| OAHl | -0.081 | 0.413 | SpO ₂ nadir | 0.057 | 0.574 |
| Model 3 Dependent variable: ln MMP-9 p=0.017 | | | Model 4 Dependent variable: ln MMP-9 p=0.018 | | |
| Parameters | β | p values | Parameters | β | p values |
| Age | 0.027 | 0.779 | Age | 0.032 | 0.748 |
| Gender | 0.122 | 0.212 | Gender | 0.128 | 0.199 |
| BMI z-score | 0.223 | 0.027 | BMI z-score | 0.155 | 0.121 |
| CRP | 0.257 | 0.008 | CRP | 0.263 | 0.008 |
| OAHl | -0.083 | 0.392 | SpO ₂ nadir | 0.038 | 0.699 |

Abbreviations

| | |
|------------------------|---|
| BMI | Body mass index |
| CRP | C-reactive protein |
| MMP-9 | Matrix metalloproteinase-9 |
| OAHl | Obstructive apnea-hypopnea index |
| SpO₂ | Oxygen saturation of hemoglobin by pulse oximetry |

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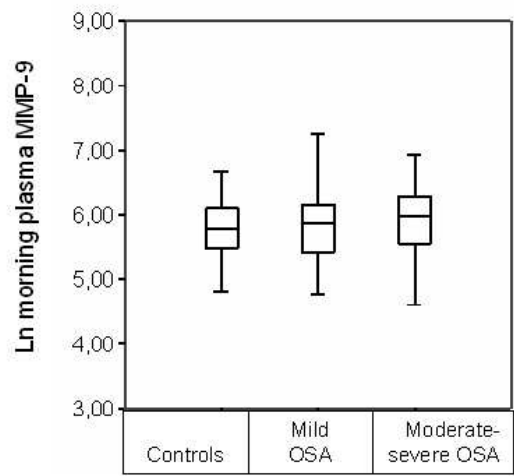


Figure 1

145x227mm (96 x 96 DPI)

RESPONSE TO REVIEWERS' COMMENTS

Reviewer 2

1. **“These authors investigated MMP-9 levels in plasma, however they did not investigate MMP-9 activity using specific protease assays. It is quite plausible that while the MMP-9 levels did not differ amongst different severities of OSA, MMP-9 activity may differ, and reflect potentially different levels of TIMPs (tissue inhibitors of metalloproteinases). In previous studies, both MMP-9 concentrations and activity levels were elevated in adults with OSA.”**

This limitation has been discussed in the revised manuscript:

“Finally, only plasma MMP-9 levels but not activity of the enzyme were measured. While the three groups of study participants did not differ in terms of MMP-9 concentrations, it is plausible that they were different regarding MMP-9 activity due to variable levels of tissue inhibitors of TIMPs.” (p. 11, par. 1)

2. **“Also, there is no mention of inclusion or exclusion of children with hypertension or any other cardiovascular disease, or children on agents that alter cardiovascular status (sympathomimetics, etc). Since children with altered vasomotor tone may potentially have altered inflammatory profiles, it is important this potential confounder be discussed.”**

None of the participants had a diagnosis of cardiovascular disease or was using sympathomimetics. This important point was clarified in the revised paragraph listing the exclusion criteria for participation in the study. (p. 4, par. 2)

3. **“Similarly, children with altered metabolic profiles secondary to OSA or obesity may have differing degrees of inflammation which may reflect in MMP-9 levels. Was this investigated by this group? If not, the authors need to mention that the potential of metabolic disease, or the presence of the metabolic syndrome for that matter, may have influenced the study’s findings.”**

Please also see our response to comment #4. Although some of the studied children were obese, our participants could be mostly classified to the “classical” pediatric OSA phenotype. In this phenotype, insulin resistance and dyslipidemia due to sleep apnea are infrequent.

4. **“Finally, were all children with OSA who were undergoing polysomnography for snoring also found to have adenotonsillar hypertrophy, or were there children who had undergone adenotonsillectomy with residual sleep disordered breathing. Certainly there is emerging evidence that there are two variants or types of sleep apnea in children. There appears to be an older, obese phenotype that does not respond to adenotonsillectomy, and**

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3 **these children resemble the adult phenotype. The classical phenotype of**
4 **young children who respond to adenotonsillectomy, fit a potentially different**
5 **inflammatory phenotype. As such I believe the authors need better**
6 **descriptive data of their 84 children with OSA to draw conclusions and make**
7 **use of the data.”**
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11 It was clarified in the revised Methods section that all children with OSA had
12 adenotonsillar hypertrophy and were studied prior to adenotonsillectomy. In
13 addition, the majority of our children were younger than 10 years old and
14 probably belonged to the “classical” pediatric OSA phenotype. The Discussion
15 section was expanded to include the important comment made by the reviewer:
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18 “Third, there is emerging evidence for two variants or types of pediatric OSA.⁴¹
19 There appears to be a phenotype in older and obese children and adolescents
20 resembling sleep apnea in adults, with a low-grade systemic inflammatory
21 response, insulin resistance, dyslipidemia and inadequate improvement after
22 adenotonsillectomy. The majority of participants of the present study were
23 younger than 10 years old and probably belonged to the classical sleep apnea
24 phenotype with adenotonsillar hypertrophy, less intense systemic inflammatory
25 response and apparent improvement after adenotonsillectomy.” (p. 10, par. 1)
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- 28 **5. “In my opinion, the figure 1 is redundant and not needed. Also, the authors**
29 **do not explicitly state a hypothesis.”**
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31
32 A hypothesis has been included in the revised manuscript:

33 “It was hypothesized that children with OSA have increased MMP-9 levels.” (p.
34 4, par. 1)
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38 **6. “Grammatical Errors:**
39 **Page 2, Line 13 should read: The Aim of this investigation**
40 **Page 3, Line 25: The first entry of MMP-9 should not be abbreviated**
41 **Page 3, Line 58: The first entry of US children should not be abbreviated.”**
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44 Grammatical errors were corrected.
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7 Adiposity and Low-Grade Systemic Inflammation Modulate
8 Matrix Metalloproteinase-9 Levels
9 in Greek Children with Sleep Apnea
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47 **Funding sources:** University of Thessaly Research Committee
48

49 **None of the authors has any conflicts of interest to disclose**
50

51 **Running title:** MMP-9 and sleep apnea
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Abstract

Background: Matrix metalloproteinase-9 (MMP-9) plasma levels correlate with C-reactive protein (CRP) concentrations and they are both increased in adults with obstructive sleep apnea (OSA). No studies have evaluated MMP-9 levels in children with sleep apnea and CRP is not consistently elevated in pediatric OSA. ~~The aim of~~ this investigation was to evaluate the association of severity of OSA, adiposity and CRP with MMP-9 plasma levels in Greek children.

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Methods: Consecutive children with snoring who underwent polysomnography and were found to have OSA (obstructive apnea-hypopnea index-OAHI ≥ 1 episode/hour) were recruited. Subjects without OSA (OAHI < 1 episode/hour) were included for comparison. Morning plasma MMP-9 and CRP were measured.

Results: Twenty nine children with moderate-to-severe OSA (age 5.4 ± 1.5 years; OAHI 13.9 ± 13.0 episodes/hour), 55 participants with mild OSA (6.4 ± 2.6 years; OAHI 2.4 ± 1.1 episodes/hour) and 22 subjects without OSA (6.8 ± 2.6 years; OAHI 0.6 ± 0.2 episodes/hour) were studied. Children with moderate-to-severe OSA were similar to those with mild OSA or without OSA regarding ln-transformed MMP-9 values (5.87 ± 0.60 vs. 5.84 ± 0.55 vs. 5.80 ± 0.46 ; $p > 0.05$) and CRP concentrations (0.22 ± 0.29 mg/dL vs. 0.21 ± 0.36 vs. 0.13 ± 0.16 mg/dL; $p > 0.05$). In multiple linear regression, body mass index ($p = 0.027$) and CRP levels ($p = 0.008$), but not OAHI or SpO₂ nadir ($p > 0.05$), were significantly related to MMP-9 values.

Conclusions: Adiposity and systemic inflammation unrelated to OSA severity, modulate MMP-9 levels in Greek children.

Introduction

Obstructive sleep-disordered breathing (SDB) with its associated nocturnal intermittent hypoxemia is considered an oxidative stress disorder characterized by release of inflammatory mediators, increased leukocyte adherence to endothelial cells, reduced nitric oxide availability and ultimately endothelial dysfunction and vascular injury.¹⁻³ Systemic inflammation and monocyte migration into the vascular wall have central roles in the pathogenesis of atherosclerosis and can potentially explain the epidemiologic association between obstructive sleep apnea and risk of cardiovascular events.⁴⁻⁶

Matrix metalloproteinase-9 (MMP-9) contributes to degradation of the extracellular matrix and to vascular remodelling and its plasma levels correlate with indices of systemic inflammation like C-reactive protein (CRP).^{7,8} Studies in adult sleep apneics have associated CRP and MMP-9 serum levels with severity of SDB and with the degree of adiposity.⁹⁻¹² In addition, monocytes isolated from adults with severe sleep apnea have higher production of MMP-9 than monocytes from control subjects.¹³ Treatment of sleep apnea with nasal continuous positive airway pressure diminishes serum levels of MMP-9 and its production from monocytes.^{11,13}

There are no published studies assessing MMP-9 plasma concentrations in children with obstructive SDB. It is expected that increased levels of CRP will be accompanied by elevated concentrations of MMP-9.⁸ However, the strength of the association between severity of intermittent upper airway obstruction during sleep and CRP in childhood may vary according to the ethnic population studied.¹⁴⁻¹⁹ It is known that Greek and Australian children with SDB have lower serum CRP concentrations than United States (US) children with SDB of similar severity.

^{14,17,19,20} Thus, the aim of this investigation was to assess whether severity of

Deleted: matrix metalloproteinase-9 (

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2 intermittent upper airway obstruction during sleep, morning plasma concentration of
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4 CRP and degree of adiposity are significantly related to morning plasma MMP-9
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6 levels in Greek children with OSA. It was hypothesized that children with OSA have
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8 increased MMP-9 levels.

11 Patients and Methods

13 Participants and clinical evaluation

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15 The study protocol was approved by the Larissa University Hospital Ethics
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17 Committee and parents of participants provided informed consent. Consecutive
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19 children with history of snoring and adenotonsillar hypertrophy, who underwent
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21 polysomnography and were found to have obstructive sleep apnea (OSA) were
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23 recruited in the study prior to adenotonsillectomy. OSA was defined as an obstructive
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25 apnea-hypopnea index (OAH) ≥ 1 episode/hour. Subjects with hypertrophic tonsils
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27 and without snoring, who underwent polysomnography to rule out OSA and who had
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29 OAH < 1 episode/hour were included in the study for comparison (group without
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31 OSA). Exclusion criteria for participation in the study were: i) symptoms or signs of
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33 acute or chronic inflammation (e.g. recurrent tonsillitis); ii) history of cardiovascular,
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35 neuromuscular or genetic disorders; and iii) use of sympathomimetics, corticosteroids
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37 or leukotriene modifiers.

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39 Study participants underwent a detailed physical examination with
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41 measurement of weight and standing height. Body mass index (BMI) and BMI z-
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43 score were calculated. Obesity was defined as BMI z-score > 1.645 .²¹

45 Polysomnography

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47 Participants underwent overnight polysomnography in the Sleep Disorders
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49 Laboratory of Larissa University Hospital. The following parameters were recorded:
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51 electroencephalogram (C3/A2, C4/A1, O1/A2); right and left oculogram; submental
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2 and tibial electromyogram; body position; electrocardiogram; thoracic and abdominal
3 wall motion; oronasal airflow (3-pronged thermistor); and oxygen saturation of
4 hemoglobin.
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8 Arousals were scored according to recommendations provided in the
9 American Academy of Sleep Medicine Manual.²² Obstructive apnea was defined as
10 a >90% fall in the airflow signal amplitude compared to the pre-event baseline, lasting
11 for at least 2 missed breaths and in the presence of continued or increased inspiratory
12 effort.²² An apnea was considered as mixed if part of the event was associated with
13 absent inspiratory effort and the remaining part with the presence of respiratory
14 movements. Hypopnea was scored when there was a decrease in the airflow signal
15 amplitude of at least 50% compared to the pre-event baseline, that lasted for at least 2
16 missed breaths and which was associated with an arousal or $\geq 3\%$ desaturation.²²
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18 OAHl was equal to the average number of obstructive and mixed apneas and
19 hypopneas per hour of sleep.
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30 **Measurement of plasma MMP-9 and CRP**

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32 Venipuncture was completed in the morning after polysomnography. The
33 blood sample was collected into a tube containing heparin and was immediately
34 centrifuged. Part of the supernatant (platelet-free plasma) was aliquoted and frozen at
35 -70°C for later measurement of MMP-9 concentration, and the remaining sample was
36 processed for determination of CRP level.
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42 A high sensitivity immunonephelometric method with lowest detection limit
43 of 0.0175 mg/dL (N High Sensitivity CRP; Dade Behring, Marburg, Germany) was
44 used for quantification of CRP levels. MMP-9 concentration was measured with a
45 commercially available solid phase ELISA (Quantikine MMP-9 Immunoassay; R&D
46 Systems Inc., Minneapolis, MN). The assay has a lowest detection limit of 0.156
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2 ng/mL, intra-assay coefficient of variation 1.9%-2.9% and inter-assay coefficient of
3
4 variation 6.9%-7.9%.

6 **Data analysis**

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8 Morning plasma MMP-9 level was the primary outcome measure of the
9
10 current investigation. MMP-9 concentrations were log-transformed (natural
11
12 logarithm) to approach a normal distribution. Three study groups were formed: i)
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14 subjects without OSA (OAHI < 1 episode/hour); ii) children with mild OSA (OAHI =
15
16 1-5 episodes/hour); and iii) subjects with moderate-to-severe OSA (OAHI > 5
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18 episodes/hour).
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21 The 3 study groups were compared in terms of subjects' characteristics,
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23 polysomnography indices, CRP and MMP-9 plasma levels. One-way analysis of
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25 variance (ANOVA) followed by post-hoc tests (Bonferroni's test) was carried out for
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27 comparisons of study groups regarding continuous variables and χ^2 test (Yate's
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29 correction) for comparisons regarding categorical characteristics. Analysis of
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31 covariance was also used to compare the 3 study groups in terms of ln-transformed
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33 MMP-9 taking under consideration the fact that these groups differed significantly in
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35 BMI z-score.

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37 Univariate Pearson's correlation was applied to evaluate the association of ln-
38
39 transformed MMP-9 with BMI z-score, polysomnography indices, and CRP.
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41 Multiple linear regression analysis was completed to assess whether age, gender, BMI
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43 z-score, and OAHI or SpO₂ nadir were related to ln-transformed MMP-9
44
45 concentrations. Multivariable analysis was repeated after including CRP as an
46
47 additional independent variable in the regression analysis model.
48

49 **Results**

51 **Subjects' characteristics and polysomnography findings**

Eighty four children with OSA and 22 subjects without OSA were recruited.

The 3 study groups did not differ in terms of age and gender. Ninety eight (92.5%) of all children were younger than 10 years old. Participants with moderate-to-severe

OSA had significantly higher BMI z-score than participants without OSA (Table 1).

Polysomnography findings for each study group are summarized in Table 1.

Morning concentrations of MMP-9 and CRP

Children with moderate-to-severe OSA, mild OSA or without OSA were similar regarding MMP-9 and CRP plasma concentrations (Table 2 and Figure 1).

Analysis of covariance confirmed that the 3 study groups did not differ in ln MMP-9 values even after adjustment for BMI z-score ($p=0.833$). Adjusted mean (\pm standard deviation) ln-transformed MMP-9 values for subjects with moderate-to-severe OSA, mild OSA or without OSA were: 5.85 ± 0.54 , 5.81 ± 0.54 or 5.90 ± 0.58 , respectively.

There was no correlation of ln-transformed MMP-9 levels with OAHl ($r= -0.03$; $p=0.779$), respiratory arousal index ($r= -0.07$; $p=0.456$), oxygen desaturation of hemoglobin index ($r= -0.04$; $p=0.703$) or SpO₂ nadir ($r=0.03$; $p=0.733$). In contrast, ln-transformed MMP-9 levels were significantly associated with BMI z-score ($r=0.21$; $p=0.032$) and CRP ($r=0.28$; $p=0.004$). In multiple regression analysis, BMI z-score and CRP level-but not severity of SDB-were related to ln-transformed morning MMP-9 concentrations (Table 3).

Discussion

In the present study it has been demonstrated that low-grade systemic inflammation, as reflected by CRP, and degree of adiposity affect MMP-9 plasma levels in Greek children with OSA, whereas these levels are not modulated by severity of OSA. This finding is in contrast to the reported association of MMP-9

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2 with severity of OSA in adults but in line with the previously described low CRP
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4 serum levels in Greek children with sleep apnea. ^{11,13,17,18} The absence of a
5
6 relationship between MMP-9 and polysomnography indices in Greek children
7
8 supports further the concept that different ethnic pediatric populations with OSA
9
10 undergo activation of diverse inflammatory and oxidative stress pathways.
11

12 Matrix metalloproteinases (MMPs) are a family of zinc-dependent
13
14 endopeptidases that degrade extracellular matrix proteins. ²³ MMPs are excreted as
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16 zymogens that are activated by proteinases and their action is modified by tissue
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18 inhibitors (TIMPs). ^{24,25} They are involved in the inflammatory processes related to
19
20 atherosclerosis and plasma concentration of MMP-9 is a predictor of cardiovascular
21
22 mortality in patients with coronary disease. ^{26,27} More specifically, MMPs promote
23
24 degradation of the vascular basement membrane allowing monocyte infiltration and
25
26 smooth muscle cell migration into the vascular wall, ultimately resulting in vascular
27
28 remodeling and atheromatic plaque formation. ^{7,28,29} Furthermore, MMP-9
29
30 expression in the myocardium increases in parallel with the growing left ventricle
31
32 myocardial mass in patients with ventricular pressure overload. ³⁰
33

34 Three studies in adults with OSA have identified increased serum MMP-9
35
36 concentrations or enhanced MMP-9 production by monocytes. ¹¹⁻¹³ Tazaki et al
37
38 reported that MMP-9 serum levels and enzyme activity measured by gelatin
39
40 zymography were higher in men with moderate-to-severe OSA than in patients with
41
42 mild OSA or in obese controls. ¹¹ MMP-9 levels and activity were positively
43
44 correlated with BMI and serum concentrations of interleukin-6 (IL-6) or tumor
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46 necrosis factor-alpha (TNF-alpha) and these levels decreased after treatment with
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48 nasal continuous positive airway pressure.
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A variety of cells including monocytes, macrophages, neutrophils and endothelial cells release pro-MMP-9 after stimulation by inflammatory cytokines like IL-6 and TNF-alpha.^{7,31} Hence, it is possible that hypoxemia-triggered production of IL-6 and TNF-alpha in sleep apneics promotes synthesis of MMP-9.^{7,31-33} Since IL-6 also regulates the synthesis of CRP, it is not surprising that MMP-9 and CRP are significantly correlated with each other in male subjects with OSA and their levels increase with increasing severity of sleep apnea.^{12,34} Augmented production of TNF-alpha and MMP-9 from peripheral blood monocytes in severe OSA decreases following treatment with nasal continuous positive airway pressure.¹³

Similar to findings in adults,^{11,12} in the current report, CRP and the degree of adiposity were significantly associated with MMP-9 plasma concentrations. In vitro experiments involving peritoneal macrophages from rats have revealed that CRP enhances gene expression of MMP-9.³⁵ Moreover, in healthy obese and lean children and adolescents MMP-9 levels increase linearly with increasing BMI.³⁶ A two-week high fiber and low-fat diet along with daily exercise have a lowering effect on MMP-9 serum concentrations in overweight children.³⁷

Although blood pressure elevation and changes in cardiac structure and function are the main effects of pediatric OSA on the cardiovascular system,³⁸⁻⁴⁰ it is conceivable that sleep apnea is also associated with changes in collagen turnover of the vascular wall as it has been postulated for obese children and adolescents with elevated MMP-9 concentrations.^{36,37} Nevertheless, as opposed to results from investigations in adults, MMP-9 levels in this study were not affected by severity of OSA, despite the presence of a positive correlation between plasma MMP-9 and CRP concentrations.

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3 A number of reasons could explain the discrepant results of the current study
4 compared to findings of previous reports on MMP-9 levels in adult sleep apneics.

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6 First, adults with sleep apnea have more severe intermittent hypoxemia in comparison
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8 to children with SDB and this difference may be responsible for the higher prevalence
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10 of systemic inflammation in the former than in the latter. Second, OSA is usually
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12 present for more years and hence its complications are more likely frequent in adults

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14 than in young children. Third, there is emerging evidence for two variants or types of
15 pediatric OSA.⁴¹ There appears to be a phenotype in older and obese children and
16 adolescents resembling sleep apnea in adults, with low-grade systemic inflammatory
17 response, insulin resistance, dyslipidemia and inadequate improvement after
18 adenotonsillectomy. The majority of participants of the present study were younger
19 than 10 years old and probably belonged to the classical sleep apnea phenotype with
20 adenotonsillar hypertrophy, less intense systemic inflammatory response and apparent
21 improvement after adenotonsillectomy.

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23 In addition, different inflammatory and oxidative stress pathways may be
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25 activated among populations with OSA and different genetic background or

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27 environmental exposures. ⁴² US children with obstructive SDB have increased
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29 concentrations of circulating vascular endothelial growth factor (VEGF), IL-6 and

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31 CRP, whereas VEGF and CRP appear unaffected by severity of OSA in Greek

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33 children. ⁴²⁻⁴⁴ In contrast, excretion of uric acid, an important antioxidant and free
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35 radical scavenger, is accentuated in Greek children with SDB but it is not modulated

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37 by severity of OSA in US children. ⁴⁵ Finally, urine excretion of cysteinyl

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39 leukotrienes is related to severity of SDB in Greek children. ⁴⁶ Thus, genetic

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41 variability and environmental factors may be responsible for the differential oxidative
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2 stress and inflammatory responses to intermittent upper airway obstruction during
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4 sleep.⁴⁷

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6 One potential limitation of the current study is that use of nasal thermistry to
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8 monitor airflow without measurement of nasal pressure may have underestimated the
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10 frequency of hypopneas. Also, the 3 study groups were not matched in terms of BMI
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12 z-score. However, analysis of covariance confirmed that children with moderate-to-
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14 severe OSA, mild OSA or without OSA had similar MMP-9 values even after
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16 adjustment for BMI z-score. Finally, only plasma MMP-9 levels but not activity of
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18 the enzyme were measured. While the three groups of study participants did not
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20 differ in terms of MMP-9 concentrations, it is plausible that they were different
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22 regarding MMP-9 activity due to variable levels of TIMPs.
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25 The present study represents the first attempt in the pediatric literature to
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27 assess the potential association between sleep apnea and MMP-9 levels. In the study
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29 design, the sample size has not been calculated since there is no prior estimate of the
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31 variability and the clinically significant difference of MMP-9 values (primary
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33 outcome measure) in children with and without OSA. In such novel cohort studies,
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35 the number of participants is usually determined by patient availability and resources.

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37 In conclusion, MMP-9 plasma levels in Greek children with sleep apnea are
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39 affected by adiposity and by low-grade systemic inflammation unrelated to OSA.
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41 Severity of intermittent upper airway obstruction during sleep does not appear to
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43 influence MMP-9 concentrations in the studied pediatric population.
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11 **Figure 1.** Boxplots of ln-transformed matrix metalloproteinase-9 (MMP-9) plasma
12 levels in: i) children without obstructive sleep apnea (OSA) (n = 22); ii) subjects with
13 mild OSA (n = 55); and iii) children with moderate-to-severe OSA (n = 29). The
14 three groups did not differ in MMP-9 levels (p=0.736). Horizontal bars represent
15 median; whiskers represent highest and lowest values.
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