

1 **Inflammatory markers and bone mass in children with overweight/obesity: the role**
2 **of muscular fitness**

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33

Abstract

34

35 **Objectives:** To examine which inflammatory markers are associated with bone mass and
36 whether this association varies according to muscular fitness in children with
37 overweight/obesity.

38 **Methods:** Plasma interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), epidermal
39 growth factor, vascular endothelial growth factor A (VEGF) and C-reactive protein were
40 analyzed in 55 children (38 boys) aged 8 to 11 yr. Muscular fitness was computed as the
41 mean of the 1 maximum repetition in bench press and leg press standardized values. Fat
42 mass, lean mass, and bone mineral content (BMC) of the total body less head (TBLH)
43 and lumbar spine (LS) were assessed using dual-energy x-ray absorptiometry.

44 **Results:** IL-6 ($\beta = -0.136$) and VEGF ($\beta = -0.099$) were associated with TBLH BMC,
45 whilst TNF- α ($\beta = -0.345$) and IL-1 β ($\beta = 0.212$) were associated with LS BMC after
46 controlling for potential cofounders ($P < 0.05$). There was a trended interaction of
47 muscular fitness in the association of VEGF with TBLH BMC ($P = 0.122$) and TNF- α
48 with LS BMC ($P = 0.057$). Stratified analyses by muscular fitness levels showed an
49 inverse association of VEGF with TBLH BMC ($\beta = -0.152$, $P = 0.032$) and TNF- α with
50 LS BMC ($\beta = -0.491$, $P < 0.001$) in the low-fitness group, whilst no evidence of
51 association was found in the high-fitness group.

52 **Conclusion:** IL-6, VEGF, TNF- α , and IL-1 β are significantly associated with bone mass.
53 Higher muscular fitness may attenuate the adverse effect of high levels of VEGF and
54 TNF- α on bone in this population.

55 **Key words:** cytokines, growth factors, bone health, strength, childhood obesity

56 **Introduction**

57 Obesity and osteoporosis are two major global health problems with an increasing
58 prevalence and closely related to both mortality and morbidity worldwide (1,2). The belief
59 that obesity is protective against osteoporosis has recently come into question due to the
60 increasing evidence about the endocrine function and interplay between different tissues,
61 such as muscular, adipose, and bone tissue (3). Moreover, childhood is a critical period
62 for bone accretion (4) and reaching an optimal peak bone mass is considered the best
63 protective factor against future osteoporosis and fracture.

64 The link between body composition and bone health in children and adolescents
65 has been the focus of various investigations over the last few decades (5). Previous cross-
66 sectional evidence highlights a negative association between fat mass (FM) and bone
67 mass in adolescents, once lean mass (LM) is accounted for (6). In addition, Mengel et al.
68 (7) observed that boys with overweight/obesity who had an extensive body mass index
69 (BMI) gain during puberty experienced lower gains in bone outcomes. In this regard, the
70 increasing presence of fat within the bone marrow is known to affect osteoblast
71 differentiation, increasing osteoclastic activity and affecting mineralization (8).

72 The pediatric skeleton is sensitive to factors that influence bone accrual, including
73 physical activity and increased inflammatory cytokines (9). In this sense, the role that
74 inflammatory markers play in the child's skeleton has been investigated (10–12). For
75 instance, Mengel et al. (11) found that vascular endothelial growth factor was inversely
76 associated with total body bone mineral content (BMC), whereas epidermal growth factor
77 (EGF) was inversely associated with areal bone mineral density (aBMD) and apparent
78 bone mineral density at the lumbar spine (LS). Similar to adipose tissue, the skeletal
79 muscle is a secretory organ responsible for the production of several hundreds of

80 myokines in response to exercise (13). Thus, the muscle-adipose tissue axis should be
81 taken into consideration to elucidate the systemic effects of the inflammatory markers on
82 bone health.

83 Muscular fitness has been favorably associated with potential health benefits (i.e.
84 bone health, mental health, total and central adiposity, cardiovascular disease and
85 metabolic risk factors) in children and adolescents (14). Recent studies reported that the
86 association between muscular fitness and bone outcomes was explained by LM in
87 different growth stages (15–17). In addition, muscular fitness has been inversely
88 associated with c-reactive protein (CRP) in adolescents with overweight/obesity (18,19)
89 and also in prepubertal children (20). In adolescents, a clustered score of inflammatory
90 markers including CRP, C3, C4, fibrinogen, and leptin has been inversely associated with
91 muscular fitness (21).

92 To the best of our knowledge, no study has tested the role of muscular fitness in the
93 association between inflammatory markers and bone mass. Therefore, the purpose of this
94 study was twofold: 1) to identify which inflammatory markers are associated with bone
95 mass in children with overweight/obesity and, 2) to examine whether this association
96 varies according to muscular fitness levels in this population.

97 **Material and methods**

98 *Participants and study design*

99 The present cross-sectional study was developed within the ActiveBrains project
100 framework (ClinicalTrial.gov ID: NCT02295072). A detailed description of the study
101 design, purpose, methodology and inclusion/exclusion criteria has been published
102 elsewhere (22). The ActiveBrains project measured 110 prepubertal children with
103 overweight/obesity from Granada (Southern Spain). Participants were recruited from the

104 Paediatric Unit of the University Hospitals San Cecilio and Virgen de las Nieves. The
105 study protocol was approved by the Review Committee for Research Involving Human
106 Subjects at the University of Granada (Reference: 848, February 2014) and informed
107 consent was obtained from parents.

108 In this report, a total of 55 children (10.2 ± 1.2 years, 38 boys) with complete data
109 on inflammatory markers, body composition (i.e. bone, FM and LM), objectively
110 measured muscular fitness, and sexual maturation assessment were included (see
111 flowchart in Figure 1).

112 *Anthropometry and sexual maturation*

113 Body mass (kg) was measured with an electronic scale (SECA 861, Hamburg,
114 Germany). Height (cm) and sitting height were measured with a precision stadiometer
115 (SECA 225, Hamburg, Germany). BMI was calculated as: body mass (kg)/height (m^2)
116 and the participants were classified into BMI categories according to the World Obesity
117 Federation criteria (23).

118 Somatic maturity offset was assessed as years from peak height velocity (PHV)
119 from age, height and sitting height using validated algorithms for children (24).

120 *Inflammatory markers*

121 Venous blood samples were obtained between 08:00 a.m. and 09:00 a.m. by
122 venipuncture after an overnight fast (at least 12 hours) in all participants. Blood samples
123 in tubes containing EDTA were spun immediately at 1000g for 10 min. Plasma was
124 isolated and stored at -80°C until analyses. Three key cytokines analyzed in plasma were
125 included in this study: interleukin-6 (IL-6, pg/mL), interleukin- 1β (IL- 1β , pg/mL), and
126 tumor necrosis factor- α (TNF- α , pg/mL). IL-6, IL- 1β and TNF- α were quantified by
127 multiple analyte profiling technology (MILLIPLEX[®] MAP Human High Sensitivity T

128 Cell Magnetic Bead Panel, EMD Millipore Corporation, Missouri, USA) with a kit plex
129 (HCYIL6-MAG Anti-Human IL-6 Beads set, HCYIL1B-MAG Anti-Human IL-1 β Bead,
130 and HCYTNFA-MAG Anti-Human TNF- α Beads set), using one 96-Well plate with
131 sealers (Cat. HSTCMAG-28SK). The intra- and inter-assay precision coefficients of
132 variation for IL-6 were 5% and 20%, respectively, and sensitivity was 0.11 pg/mL. For
133 both, IL-1 β and TNF- α the intra- and inter-assay precision coefficients of variation were
134 5% and 15%, respectively, with a sensitivity of 0.14 pg/mL for IL-1 β , and of 0.16 pg/mL
135 for TNF- α . CRP (mg/L) was determined by turbidimetry (AU2700 Olympus Analyzer;
136 Olympus UK Ltd, Watford, UK) with a sensitivity of 0.007 mg/L and inter-assay
137 coefficients of variation 1.9%.

138 Two growth factors were analyzed by multiple analyte profiling technology
139 (MILLIPLEX[®] MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, EMD
140 Millipore Corporation, Missouri, USA) with a kit plex (HVEGF-MAG Anti-Human
141 VEGF-A Bead, and HAGEGF-MAG Anti-Human EGF Bead), using two 96-Well plates
142 with sealers (Cat. HAGP1MAG-12K). The intra- and inter-assay precision coefficients
143 of variation for vascular endothelial growth factor A (VEGF, pg/mL) were 3.5% and 10%,
144 respectively, and sensitivity was 8.1 pg/mL. For EGF (pg/mL), the intra- and inter-assay
145 precision coefficients of variation were 3.2% and 6.8%, respectively, with a sensitivity of
146 1.0 pg/mL.

147 *Body composition*

148 Children were scanned with dual-energy X-ray Absorptiometry (DXA) using the
149 Hologic Discovery Wi (Hologic Series Discovery QDR, Bedford, MA, USA). The DXA
150 equipment was calibrated at the start of each testing day by using a lumbar spine phantom
151 as recommended by the manufacturer. All DXA scans and analyses were performed using

152 the GE encore software (version 4.0.2) and were completed following the same protocol
153 by the same researcher. The positioning of the participants and the analyses of the results
154 were undertaken following recommendations from the International Society of Clinical
155 Densitometry (25). The total body scan was used to obtain FM, LM, and BMC at the total
156 body less head (TBLH) and at the LS.

157 *Objectively measured muscular fitness*

158 Muscular fitness was evaluated in laboratory conditions. We determined each
159 participant's 1 repetition maximum (1RM) when the child was able to lift throughout the
160 full range of motion in bench press and leg press tests (26). Participants received
161 familiarization sessions before the testing session in order to ensure an adequate technique
162 (i.e. controlled movements and proper breathing). Before attempting 1RM, participants
163 performed 6 repetitions with a light load and 3 repetitions with a heavier load (50–90%
164 estimated 1RM). Then, a series of single repetitions with increasing loads (0.5–2.3 kg for
165 bench press and 10–20 kg for leg press) were performed. The 1RM was determined when
166 participants fell short of the full range of motion on at least two non-consecutive attempts.
167 A resting time of 3–5 minutes between attempts was allowed. Rate of perceived exertion
168 at each attempt was obtained using the children's OMNI-Resistance Exercise scale (27).
169 Moreover, during all testing procedures researchers obtained more information from the
170 participants by asking questions such as: “How do you feel?”, “Is the load light, medium
171 or heavy?” and “Could you lift more?” to aid in the progression of the 1RM trials.

172 A muscular fitness score was computed by combining the standardized values of
173 1RM bench press and 1RM leg press tests. Each of these variables was standardized as
174 follows: $\text{standardized value} = (\text{value} - \text{mean}) / \text{SD}$. The muscular fitness z-score was
175 calculated as the mean of the 2 standardized scores (1RM bench press and 1RM leg press).

176 *Statistical analyses*

177 Data were analyzed using SPSS IBM statistics (version 20 for Windows, Chicago,
178 IL) and the normal distribution of the raw variables was confirmed using visual check of
179 histograms, Q-Q and box plots. Statistical significance was defined as $P < 0.05$. Interaction
180 analyses were performed between sex and inflammatory markers on the outcomes. No
181 significant interactions were found ($P > 0.05$), so analyses were carried out for boys and
182 girls together.

183 Descriptive characteristics of participants are presented as mean \pm standard
184 deviation (SD). Differences between sexes were determined by independent t tests.
185 Stepwise hierarchical regression analyses were carried out to identify the inflammatory
186 markers that best predicted bone mass. Sex, years from PHV and TBLH LM were
187 considered for entry into step 1 of the model, and subsequent addition of inflammatory
188 markers in step 2 was conducted to determine the contribution to the bone mass variables
189 following step 1 adjustments. These covariates were selected because of their known
190 association with bone mass (6). The standardized regression coefficients (β) are reported
191 and the squared semi-partial correlation coefficients (sr^2) were used to determine the
192 contribution of each predictor in the overall variance of the model after removing shared
193 contributions with other predictors. Collinearity was checked for the variables using the
194 variance inflation factor and tolerance levels.

195 Finally, multiple linear regression analysis with interaction effect was used to test
196 the role of muscular fitness in the association between inflammatory markers (those that
197 were significant in the stepwise regression models) and bone mass. The interaction effects
198 of muscular fitness in the association between inflammatory markers and bone mass were

199 further examined (in those with $P < 0.20$), stratifying by high/low (above/below sex-, age-
200 , and study-specific median) levels of muscular fitness.

201 **Results**

202 Descriptive characteristics are presented in Table 1 (mean \pm SD). Girls were more
203 mature than boys ($P < 0.05$) but no significant differences between sexes were found in
204 the remaining descriptive variables. The mean age of the participants was 10 ± 1.2 years
205 and they were 2.3 ± 1.0 years below PHV, overweight and obesity was evident in 30.9%
206 and 69.1% of them, respectively.

207 Table 2 shows the stepwise multiple regression analyses for identifying the
208 inflammatory markers that explained the variance in the outcome variables in children
209 with overweight/obesity. The probability of F-to-remove ≥ 0.1 was established in order
210 to identify these markers. For TBLH BMC, 88% of the variance was explained by TBLH
211 LM, years from PHV, IL-6, sex, and VEGF ($sr^2 = 0.009 - 0.135$), whilst 66% of the
212 variance in LS BMC was explained by TNF- α , years from PHV, TBLH LM, IL-1 β , and
213 sex ($sr^2 = 0.001 - 0.096$).

214 The role of muscular fitness z-score in the association of inflammatory markers
215 (those previously included in the stepwise method) and bone mass is shown in Table 3.
216 After adjusting for sex, years from PHV, and TBLH LM, the interaction effect of
217 muscular fitness showed a positive trend in the association of VEGF with TBLH BMC
218 ($P = 0.122$) and TNF- α with LS BMC ($P = 0.057$). No evidence of interaction with
219 muscular fitness was found in the remaining associations of IL-6 with TBLH BMC ($P =$
220 0.857) and IL-1 β with LS BMC ($P = 0.309$).

221 Figure 2 shows the standardized β regression slopes of VEGF with TBLH BMC
222 and TNF- α with LS BMC, according to muscular fitness levels. Stratified analyses by

223 muscular fitness levels (below/above median) showed a significant inverse association
224 between VEGF and TBLH BMC in the low muscular fitness group (Figure 2A, $\beta = -$
225 0.152, $P = 0.032$), whilst no evidence of association was found in the high muscular
226 fitness group (Figure 2A, $\beta = -0.045$, $P = 0.598$). Likewise, an inverse association between
227 TNF- α and LS BMC was found in the low muscular fitness group (Figure 2B, $\beta = -0.491$,
228 $P < 0.001$), although this association was non-significant in the high muscular fitness
229 group (Figure 2B, $\beta = -0.060$, $P = 0.666$).

230 **Discussion**

231 In the present study we showed IL-6 and VEGF to be associated with TBLH BMC
232 and TNF- α and IL-1 β with LS BMC in children with overweight/obesity. In addition, our
233 results suggested that higher levels of muscular fitness may attenuate the adverse effects
234 of VEGF and TNF- α on TBLH BMC and LS BMC, respectively. To the best of our
235 knowledge, this is one of the few studies which thoroughly addresses the influence of
236 inflammatory markers on bone mass, and the first study examining the role of muscular
237 fitness in the relationship between inflammatory markers and bone mass.

238 *Inflammatory markers and bone mass in overweight/obese children*

239 In this study, an inverse association between VEGF and TBLH BMC was found
240 after controlling for the effect of sex, years from PHV, and TBLH LM. Our results were
241 comparable to a longitudinal study in which serum VEGF was inversely associated with
242 BMC/height at the total body in boys with overweight whose BMI gain was higher during
243 pubertal years (11). Elevated circulating levels of VEGF have been found in obese
244 population as hypoxia-induced by adipose tissue expansion produce VEGF (28).
245 However, VEGF functions on bone development depend both on autocrine and paracrine
246 pathways. For instance, VEGF stimulates osteoblast differentiation and inhibits

247 adipocytes differentiation via intracrine pathway, whereas osteoblast-derived VEGF
248 leads osteoclast differentiation via paracrine pathway (29). In the light of these findings,
249 we could speculate that despite VEGF concentrations are important for the adipose tissue
250 vascularization in this population, highly-expressed VEGF as a consequence of the
251 overweight/obese condition might have detrimental effects on bone mass accumulation,
252 possibly explained by the dysregulation of autocrine and paracrine mechanisms.

253 IL-6 was also inversely associated with TBLH BMC after controlling for the same
254 set of cofounders. Our results are in accordance with Hanks et al. (10) who found a
255 negative correlation between IL-6 and BMC in prepubertal girls. Similarly, Mengel et al.
256 (11) found that the changes in serum IL-6 were negatively correlated with LS aBMD in
257 boys with overweight and extensive BMI gain during the pubertal years. However, they
258 did not find any association between IL-6 and LS aBMD after controlling for the effect
259 of testosterone, body fat percentage, and BMI. Previous studies have reported that IL-6
260 directly promotes osteoclastogenesis by binding with receptors on pre-osteoclasts or
261 indirectly alters bone remodeling by inducing JAK/STAT3 pathways through osteoblasts
262 and secrete pro-osteoclasts mediators (i.e. receptor activator of nuclear factor kappa-B
263 ligand [RANKL], and IL-1) (30). Overall, our findings agree with the idea that IL-6 have
264 anti-osteogenic and pro-osteoclastic effects on bone and these effects might already be
265 present in prepubertal children with overweight/obesity.

266 Like IL-6, *in vitro* studies have also reported the osteoclastogenic role of TNF- α
267 (31). Otherwise, few studies have documented this role in humans. Zheng et al (32)
268 reported that TNF- α produced by stimulated whole blood cells was inversely associated
269 with LS aBMD in postmenopausal women, whereas Ding et al (33) found that the inverse
270 association between serum TNF- α and LS aBMD in older men disappeared after
271 controlling for IL-6. Our results indicate that plasma levels of TNF- α were inversely

272 associated with LS BMC independently on sex, years from PHV, and TBLH LM. Besides,
273 IL-6 did not come up as a predictor of LS BMC in the stepwise multiple linear regression.

274 IL-1 β is a proinflammatory marker associated with osteoclastogenesis via induction
275 of RANKL and inhibition of osteoprotegerin and reduces osteoblast recruitment *in vitro*
276 (34). Unexpectedly our results suggest, for the first time, a positive association between
277 IL-1 β and LS BMC after controlling for potential cofounders in children with
278 overweight/obesity. This finding agrees with the study of Pacifici et al. (35) in which an
279 increased IL-1 production was associated with bone formation in adults independently on
280 sex and menopausal status. This can be explained by the fact that short stimulation of
281 mesenchymal stem cells with IL-1 β leads to osteogenic differentiation through
282 upregulation of genes in MG63-GFP osteoblasts (36). On the contrary, IL-1 β has been
283 negatively associated with LS aBMD in postmenopausal women (32) and Mengel et al.
284 (11) did not find significant associations between IL-1 β and aBMD in overweight
285 children.

286 *Muscular fitness, inflammatory markers and bone mass in overweight/obese children*

287 Bone marrow is a complex environment, in which a variety of cell types (i.e. blood
288 cells, osteoblasts, osteoclasts, and adipocytes) share a common space locally releasing
289 cytokines and growth factors that could affect the cells in their proximity (8).
290 Furthermore, bone development is regulated by modelling and remodeling processes that
291 depend on the mechanical forces applied by the muscles to the skeleton (37). Our study
292 suggests that higher levels of muscular fitness might attenuate the detrimental effects of
293 VEGF and TNF- α on TBLH BMC and LS BMC, respectively (Figure 2). In this regard,
294 partial correlations controlling for sex and years from PHV showed that VEGF was
295 negatively correlated with muscular fitness (data not shown, $r = -0.36$, $P = 0.008$),

296 although no evidence of correlation was found between TNF- α and muscular fitness (data
297 not shown, $r = -0.01$, $P = 0.923$). The latter result contrasts with Steene-Johannessen et
298 al. (20) who found a negative correlation between TNF- α and muscular fitness in
299 prepubertal children after controlling for pubertal stage. Nevertheless, the beneficial
300 effect of muscular fitness on bone development is well documented in growing children
301 (38). Moreover, the association between muscular fitness and bone mass is mediated by
302 LM in prepubertal children (15). Likewise, an inhibitory effect of LM on obesity-related
303 inflammation has been suggested in middle-aged adults (39). Thereby, our finding agrees
304 with literature and supports the fact that there is a crosstalk between adipocytes and
305 myocytes interacting with obesity and its related-disorders even in children with
306 overweight/obesity. We speculate that the detrimental consequences of excessive FM (i.e.
307 inflammation) in children with overweight/obesity could be counteracted, to some extent,
308 by maintaining optimal levels of muscular fitness.

309 **Strengths and limitations**

310 Some limitations need to be considered. At first, our cross-sectional design rules
311 out the possibility of identifying cause-effect relationships. Secondly, the number of
312 participants with complete data in all studied variables is relatively small, but similar to
313 previous studies (11,12). Thirdly, our study has used plasma samples to measure
314 inflammatory markers. Previous studies have used plasma or serum samples and
315 therefore, comparisons may be affected. However, as shown in a recent study the
316 correlations between plasma and serum measurements suggest that the differences in
317 metabolite concentrations does not necessarily introduce a bias in cross-sectional studies
318 (40). Notwithstanding, the use of DXA and the accuracy of the objective methodology
319 used for muscular fitness and blood measurements are strengths of this study.

320 **Conclusion**

321 In summary, our findings suggest that the link between obesity and bone health may
322 be at least explained by inflammatory mechanisms in children with overweight/obesity.
323 Specifically, IL-6 and VEGF were negatively associated with TBLH BMC, whereas
324 TNF- α (negatively) and IL-1 β (positively) were associated with LS BMC. Furthermore,
325 our data suggest that high levels of objectively measured muscular fitness may attenuate
326 the adverse effects of VEGF and TNF- α on TBLH BMC and LS BMC, respectively. In
327 the light of these findings, appropriate levels of muscular fitness may preserve normal
328 bone accretion in this population. Future longitudinal and intervention studies in this
329 population are needed to confirm these findings.

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348 **Compliance with ethical standards**

349 **Conflict of interest** The authors declare that they have no conflict of interest

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475 **Figure 1.** Flowchart of study participants.

476 DXA dual-energy x-ray absorptiometry; 1RM one repetition maximum.

477

478 **Figure 2.** Graphical representation of the standardized regression slopes between VEGF

479 and TBLH BMC by levels of muscular fitness (A); and between TNF- α and LS BMC

480 by levels of muscular fitness (B). High/low fitness groups were defined as being

481 above/below the age, sex and study-specific median values for average muscular fitness

482 z-score[†]. The regression models were adjusted for sex, years from PHV, and TBLH LM.

483 The standardized coefficients are interpreted as the number of SDs that the outcome

484 changes as a result of 1-SD change in the predictor.

485 [†] Z-score mean computed from 1RM bench press (kg) and 1RM leg press (kg) tests

486 PHV peak height velocity; VEGF vascular endothelial growth factor A; TNF- α tumor

487 necrosis factor alpha; TBLH total body less head; BMC bone mineral content; LM lean

488 mass