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PAPER

# Inflammatory mediators in overweight and obese Spanish adolescents. The AVENA Study

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**OBJECTIVE:** The aim of this study was to clarify if there is an association between overweight and a state of chronic, low-grade inflammation in adolescents.
**DESIGN:** The study is a part of the cross-sectional multicenter study AVENA, designed to evaluate the nutritional status of a representative sample of Spanish adolescents. The adolescents were divided into two groups: (1) nonoverweight and (2) overweight/obesity using the International Obesity Task Force (IOTF) standards.
**SUBJECTS:** A geographically representative subsample of the AVENA study including 493 Spanish adolescents, aged 13–18 y (236 females/257 males), participated in this study.
**MEASUREMENTS:** Serum C-reactive protein (CRP) and *in vitro* production of interleukin 6 and tumor necrosis factor $\alpha$ were measured, together with a detailed anthropometry.
**RESULTS:** The inflammatory markers showed generally higher values in subjects with overweight/obesity than in those with nonoverweight, with only CRP showing significant differences (the means were 0.83 and 1.27 mg/l in the nonoverweight and overweight/obesity groups, respectively).
**CONCLUSION:** Although we have not studied if adolescent overweight and obesity play an initiating role in the development of future diseases, we suggest it may induce a chronic low-grade inflammatory state, which points out the importance of maintaining an appropriate body weight, to avoid obesity-related diseases in adulthood.
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**Keywords:** adolescents; inflammation; C reactive protein; interleukin-6; tumor necrosis factor-$\alpha$

## Introduction

Obesity has been suggested to be associated with a state of chronic, low-grade inflammation. This condition is characterized by abnormal cytokine production, increased acute-phase reactants and activation of inflammatory signaling pathways.[1,2] Childhood and adolescent obesity lays the metabolic groundwork for adult cardiovascular disease and type 2 diabetes, since risk factors such as dyslipidemia, hypertension, hyperinsulinemia and obesity often coexist in children and adolescents.[3] The metabolic syndrome has already been observed in children and adolescents,[4] and has been associated with several inflammatory markers in adults.[5] Evidence suggests that cardiovascular diseases involve inflammatory processes and that immune response may also play an initiating early role in the development of the cardiovascular lesion. The presence of early precursors of atherosclerosis and early-stage atherosclerotic lesions has been documented in children and young adults,[6–9] but studies regarding inflammatory markers in children and adolescents with overweight/obesity are rather scarce. Thus, the aim of this study was to clarify if there is an association between overweight and a state of chronic, low-grade inflammation in adolescents.

## Methods

### Study design

The complete and detailed methodology of the project has been described elsewhere.[10] This research was conducted in the context of the multicenter study AVENA (Alimentación y Valoración del Estado Nutricional en Adolescentes: Food and Assessment of the Nutritional Status of Adolescents), designed to evaluate the nutritional status, immunocompetence, body composition, lipid-related genetic markers, and

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physical activity and fitness in a representative sample of Spanish adolescents from five geographically different cities in Spain, in order to identify risk factors for chronic diseases in adulthood. All of the subjects fulfilled the inclusion criteria of the study. After receiving complete information about the aims and methods of the study, all subjects and their parents or guardians signed fully informed written consent. The protocol was approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

### Study population

A total of 493 adolescents (236 female and 257 male) ranging form 13 to 18 y were recruited from public and private schools. Before blood sampling, each subject was evaluated and all anamnesis data were registered and reviewed to judge the adolescent's medical status. Only healthy adolescents were included in this study. Thus, subjects presenting chronic diseases thought to have possible effect on immune function (eg asthma) or taking any medications with known immunological effects were excluded from the study. Subjects with any acute medical conditions, such as minor infections (upper respiratory illness), were also excluded.

### Anthropometric methods

For anthropometric measurements, subjects were barefoot and in their underwear. Weight was measured with a Seca scale (with a precision of $\pm 100$ g), height with incorporated stadiometer to the scale. Skinfold thickness (biceps, triceps, subscapular, suprailiac) was measured with a Holtain lipocaliper (0–40 mm) following standard procedures for adolescents, and the sum of the skinfolds was used to reflect adiposity between groups. Harmonization and standardization of anthropometric measurements used to assess body composition within the AVENA multicenter study was strictly controlled and has been previously published by Moreno *et al*,[11] and the AVENA group. The International Obesity Task Force (IOTF) has proposed the use of international cutoff points in children and adolescents to be used as reference standards for body mass index, and the subjects were divided into nonoverweight, overweight and obesity, according to the IOTF criteria published by Cole *et al*.[12] To achieve groups with higher numbers of subjects, the overweight and obesity groups were studied as one group. During anthropometrical measurements, trained interviewers asked the adolescents to classify themselves in one of the five stages of pubertal maturity defined by Tanner and Whitehouse.[13] The standard staging of pubertal maturity describes breast and pubic hair development in girls and genital and pubic hair development in boys.

### Laboratory methods

Standardized in all cities, blood samples were collected by venipuncture by a trained phlebotomist between 0800 and 0930. Blood for serum samples was collected in vacumtainers and allowed to clot. The serum was separated by centrifugation, divided into aliquots, and frozen and stored at $-80^{\circ}$C until withdrawn for analysis. Serum C-reactive protein (CRP) concentrations were measured by nephelometry (Sanilab, SA, Madrid, Spain). Cytokine production was assessed in cultured mitogen-stimulated peripheral blood mononuclear cells (PBMC). Mononuclear cells were isolated from heparinized peripheral blood in Ficoll–Hypaque (Lymphoprep, Hyegaard, Oslo, Norway) and washed twice in RPMI-1640 medium (BioWhittaker, Verviers, Belgium). The PBMC were resuspended in RPMI-1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin. The concentration was adjusted to $10^6$ viable cells/ml and 1 ml of cell suspension was incubated per well with mitogens, phytohemagglutinin (3.5 μl/ml) and lipopolysaccharide (1.5 μl/ml), in 24-well plates for 48 h, at 37°C and 5% $CO_2$. Following incubation the cells were removed by centrifugation and supernatant stored at $-80^{\circ}$C prior to analysis. Cytokine (interleukin (IL-6) and tumor necrosis factor α (TNF-α)) content of the supernatant was assessed using the Human Th1/Th2 cytokine CBA II kit (BD Biosciences Pharmingen, San Diego, CA), and analyzed by flow cytometry.

### Statistical analysis

The sample was adjusted by a weight factor in order to equilibrate the sample in accordance to the distribution of the Spanish population (Source: The Spanish National Institute for Statistics) and to guarantee the real representativeness of age and gender. Statistical analyses were performed using the SPSS statistical software release 11.5 for Windows XP. Student's *t*-test was used for comparison between the non-overweight and overweight/obesity groups. For statistical analyses, CRP concentration was log transformed to improve the distribution of this variable. Statistical significance was set at $P<0.05$.

## Results

Anthropometrical study and concentrations of CRP, IL-6 and TNF-α were available from 493 adolescents within the AVENA study, and were therefore included in this study. In all, 47.9% ($n=238$) were females and 52.1% ($n=257$) males. As CRP concentrations $>10$ mg/l may reflect an acute-phase response to infectious disease or disorders characterized by acute inflammation, we excluded nine subjects from the final sample (six nonoverweight and three overweight).

The mean age ($\pm$s.e.) of the studied group of participants were $15.33\pm0.08$ y in females and $15.40\pm0.09$ y in males. In the analyzed sample of the AVENA study, including immunological data, females showed a prevalence of 17% of

**Table 1** Characteristics of the overweight and nonoverweight groups

| | *Nonoverweight* | *Overweight and obesity* | P-*value*[a] |
|---|---|---|---|
| *Age* | | | |
| Females | 15.46±0.11 | 15.01±0.21 | NS |
| Males | 15.34±0.11 | 15.42±0.16 | NS |
| *BMI (kg/m$^2$)* | | | |
| Females | 20.46±0.13 | 26.87±0.52 | <0.005 |
| Males | 20.15±0.15 | 26.63±0.35 | <0.005 |
| *Sum of skinfolds*[b] *(mm)* | | | |
| Females | 47.04±0.96 | 82.16±3.43 | <0.005 |
| Males | 32.53±0.83 | 73.39±2.68 | <0.005 |

Means±s.e.; NS, not significant. [a]Student's *t*-test (statistical significance was set at $P<0.05$). [b]Biceps, triceps, subscapular and suprailiac.

overweight and 4% of obesity; the figures for males were 22 and 8%, respectively. Detailed information of overweight and obesity prevalence within the complete AVENA study is described elsewhere in this supplement by Moreno *et al*.[14] Characteristics of the two groups are shown in Table 1. There were no age differences between groups; however, as expected, we found significantly higher BMI and sum of skinfolds, reflecting more adipose tissue in the overweight/obesity groups.

According to pubertal maturity, the studied adolescents were mainly at stage IV (47.9%) and stage V (36.5), with a smaller percentage at lower stages: I (0%); II (3.7%) and III (11.9%). The non-overweight group of females was at stages: II (5.7%); III (16.6%); IV (49.1%) and V (28.5%), while the overweight/obesity group of females was at stages: II (0%); III (7.2%); IV (45.4%) and V (47.4%). The stages at the nonoverweight group of males was: II (5.7); III (14.4%); IV (37.2%) and V (42.7%), while the overweight/obesity group was at stages: II (1.7); III (8.4%); IV (58.7%) and V (31.2%).

The mean values (±s.e.), medians and ranges of the 95% confidence intervals of the studied parameters for the entire sample are shown in Table 2. Significant gender differences were found in CRP ($P<0.05$), with males showing higher values. Owing to the gender differences, both in overweight and obesity prevalence and in CRP values, females and males were also studied separately. In Table 3, CRP, IL-6 and TNF-α values (mean±s.e.) are shown in the nonoverweight and overweight/obesity groups of adolescents, together with *P*-values of Student's *t*-test. The overweight/obesity group had significantly higher CRP values, both in the sample as a whole as separated by gender. Although IL-6 levels increase in the overweight/obesity group it did not reach statistical significance. In the sample as a whole there was a 12% increase, an increase that was expressed in both genders (in females by 10% and in men by 15%). Neither did the differences in TNF-α levels reach statistical significance, but we found 21% higher values in the overweight/obesity group than the values in the nonoverweight group, due to a 30% increase in males, and a negligible decrease (1%) in females.

**Table 2** Mean values (±s.e.), medians and ranges of the 95% confidence interval of the studied parameters for the entire sample and genders separately

| | *Mean±s.e.* | *Medians* | *Ranges (95% CI)* |
|---|---|---|---|
| *CRP (mg/l)* | | | |
| Females | 0.92±0.07 | 0.59 | 0.79–1.05 |
| Males | 1.31±0.10 | 0.68 | 1.12–1.51 |
| All | 1.12±0.06 | 0.62 | 1.00–1.25 |
| *IL-6 (pg/ml)* | | | |
| Females | 37 566±1543 | 32 315 | 34 526–40 607 |
| Males | 34 103±1441 | 29 205 | 31 265–36 942 |
| All | 35 827±1057 | 30 824 | 33 749–37 904 |
| *TNF-α (pg/ml)* | | | |
| Females | 2225±150 | 1684 | 1930–2520 |
| Males | 2359±141 | 1932 | 2081–2637 |
| All | 2292±103 | 1822 | 2090–2494 |

**Table 3** Serum CRP concentrations and PMNC culture production of IL-6 and TNF-α among Spanish adolescents[a], divided into nonoverweight and overweight/obesity

| | *Nonoverweight* | *Overweight and obesity* | P-*value*[b] | *Difference (%)* |
|---|---|---|---|---|
| *CRP (mg/l)* | | | | |
| Females | 0.83±0.86 | 1.27±1.41 | 0.017 | 53 |
| Males | 1.10±1.52 | 1.69±1.50 | <0.001 | 54 |
| All | 0.97±1.24 | 1.54±1.48 | <0.001 | 59 |
| *IL-6 (pg/ml)* | | | | |
| Females | 35 714±21 999 | 39 218±19 582 | 0.657 | 10 |
| Males | 33 360±20 030 | 38 331±26 476 | 0.470 | 15 |
| All | 34 533±21 033 | 38 642±24 187 | 0.469 | 12 |
| *TNF-α (pg/ml)* | | | | |
| Females | 2293±2580 | 2270±1841 | 0.812 | −1 |
| Males | 2241±1573 | 2916±3729 | 0.212 | 30 |
| All | 2226±2133 | 2691±3205 | 0.338 | 21 |

Means±s.e. [a]Subjects with blood samples included in the AVENA study (excluding participants showing concentrations >10 mg/l to avoid acute inflammation). [b]Student's *t*-test was performed after log-transforming the values.

## Discussion

The advantage of examining the associations between inflammation and obesity in adolescents is that there is no confounding by acute coronary disease. Our data support that there are inter-relationships, and showed that these are established early in life, before any cardiovascular lesion has been shown. In this representative sample of Spanish adolescents, CRP concentrations were significantly associated with overweight and obesity in both females and males. These results are in consonance with a study of a large sample of American children and adolescents, and several other studies on children,[15–18] and extend previous research showing that CRP and BMI were strongly associated in adults.[19] Cytokines are difficult to predict and to measure, and there are no reference values in order to establish their variability in a healthy population as the one tested in this

study. The values are not homogeneously distributed and it is difficult to establish their variability due to the very large ranges. No significant differences were found in cytokines between the two groups, but the mechanisms by which obesity promotes increased CRP concentration might be due to the slightly higher production of cytokines such as TNF-$\alpha$ and IL-6, induced by adipocytes, which in turn stimulates acute-phase reactant production by the liver. This effect may be reversible, because weight loss results in decreases of IL-6, TNF-$\alpha$ and CRP in adults.[20,21] However, while IL-6 and especially CRP seem to follow the adult pattern showing associations with adiposity, TNF-$\alpha$ production in childhood[22] and adolescence is less clear, and further research is necessary to clarify the involvement of this cytokine in pathologies associated with obesity in adolescence. Whether chronic low-level elevations of CRP concentrations have any direct physiologic or pathologic implication in children is unclear. In adults, however, CRP has shown significant associations with cardiovascular disease, and is considered as a cardiovascular risk factor. In pathology studies, cardiovascular diseases has been found to begin in childhood and CRP to affect the arteries of healthy children by disturbing endothelial function and promoting intima-media thickening.[23] It is important to stress that the changes in CRP levels shown in this study are always within normal ranges, which makes us consider the whole sample as healthy subjects, and the role of significantly higher CRP may be more chronic. Our study has some limitations. Owing to our cross-sectional design, we cannot establish the directionality of the associations, and also, it would be useful to know whether the measurements of CRP are stable over time. Although we asked the subjects about any clinical infection during the study and no subject with underlying causes of elevated CRP were included, we are unable to avoid that elevated CRP was not the beginning of an infection. The effect of such measurement bias should be attenuated, however, by the large numbers of participants enrolled.

In conclusion, we suggest that overweight and obesity during adolescence is associated with a chronic low-grade inflammatory response. The potential importance lies in elucidating if early immune processes may play an initiating role in the development of future risk factors, but nevertheless, this outcome points out the importance of maintaining an appropriate body weight, a healthy lifestyle and an adequate nutritional status.

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## Appendix A1. AVENA Sudy Group

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