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INFLAMMATORY STATUS IN ADOLESCENTS; THE IMPACT OF HEALTH DETERMINANTS SUCH AS OVERWEIGHT AND FITNESS

by

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Stockholm 2006

Today's adolescents are tomorrow's society.

It's our responsibility to prevent them from becoming victims of chronic diseases.

Abstract

Overweight prevalences are rising among youth and the prevention of associated chronic diseases is priority concern in public health. Inflammatory processes are involved in the pathogenesis of cardiovascular diseases and may also play an important initiating role in the development of the cardiovascular lesion. The overall aim of this thesis was to establish reference values of body fat composition in adolescence and to investigate the association between chronic low-grade inflammation and overweight, and further to examine if fitness counteracts this possible association. In total, 2160 13 to 18 year-old Spanish adolescents were studied and a subsample of 493 subjects were randomly selected for blood sampling and the study of inflammatory status. Studied parameters were: detailed anthropometry, fitness and for the evaluation of inflammatory status, serum acute phase proteins (C-reactive protein, ceruloplasmin and complement factors C3 and C4), as well as in vitro production of interleukin-6 and tumor necrosis factor-α by peripheral blood mononuclear cells. The main result of this thesis was that overweight adolescents had moderately raised inflammatory marker, which indicates that this population is in a state of chronic low-grade inflammation. Correlations between most body fat estimates and acute phase proteins suggest a pro-inflammatory action of the adipose tissue. Moreover, central obesity seems to be an independent determinant of C3 concentrations. Overweight was associated with low-grade inflammation independently of fitness status, suggesting that the modulation is mediated by body fat rather than fitness status. C3 however, was independently associated with fitness and could be a new biomarker or involved in the mechanisms of the health benefits of fitness. Whether low-grade inflammation is predicting future diseases or is merely a practical marker of body fat, is beyond the possible answers of this thesis, but nevertheless, this outcome points to the importance of maintaining an appropriate body weight to keep inflammatory status low which could eventually contribute to improve the health status of the next generation's adult population.

Key words: Adolescence, low-grade inflammation, CRP, ceruloplasmin, C3, C4, overweight, cardiorespiratory fitness.

List of Publications

I.   Moreno LA, Mesana MI, González-Gross M, Gil CM, Fleta J, Wärnberg J, Ruiz J, Sarría A, Marcos A, Bueno M and the AVENA Study Group. Anthropometric body fat composition reference values in Spanish adolescents. The AVENA Study. *Eur J Clin Nutr.* 2006;60:191–196.

II.   Wärnberg J, Moreno L, Mesana MI, Marcos A and the AVENA group. Inflammatory status in overweight and obese Spanish adolescents. The AVENA study. *Int J Obes.* 2004 Nov;28 Suppl 3:S59-63.

III.   Wärnberg J, Nova E, Moreno LA, Romeo J, Mesana MI, Ruiz RJ, Ortega FB, Sjöström M, Bueno M, Marcos A, and the AVENA study group. Inflammatory proteins are related with total and abdominal adiposity in a healthy adolescent population. The AVENA study. *Am J Clin Nutr* (revised version submitted March 2006)

IV.   Wärnberg J, Sjöström M, Ruiz J, Moreno LA, Nova E, Ortega FB, Mesa JL, Romeo J, Montero A, Marcos A and the AVENA study group. Association of Fitness and Fatness to Low-Grade Systemic Inflammation in Adolescents. The AVENA Study. *Manuscript*

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List of abbreviations

AVENA	Alimentación y Valoración del Estado Nutricional en Adolescentes [Food and Assessment of the Nutritional Status of Adolescents]
BMI	Body mass index (weight/height^2 ; kg/m^2)
VO$_2$max	Maximum oxygen consumption
CVD	Cardiovascular disease
CRP	C-reactive protein
C3	Complement factor C3
C4	Complement factor C4
IL	Interleukin
TNF	Tumor necrosis factor

1   INTRODUCTION

Overweight and obesity are widespread in adults, adolescents and children. Among youth this epidemic is rising rapidly in many countries around the world including countries in Europe as Spain and Sweden (Moreno et al. 2002, Lobstein & Frelut 2003, Ekblom et al. 2004). Childhood and adolescence are critical periods for the onset of obesity (Dietz 1997) and for obesity associated morbidity in later life (Freedman et al. 2004). Obesity is often associated to metabolic complications (Tresaco et al. 2003), psychological problems (Mustillo et al. 2003) and a reduced capacity for physical activity (Deforche et al. 2003). Obesity in youth is not only linked to direct complications but an obese child or adolescent is at risk of becoming an obese adult with an associated raised likelihood of disease and premature death (Must & Strauss 1999).

The mechanisms linking excess body fat and disease are under intensive investigation, but are still far from clear. Inflammatory processes are involved in the pathophysiology of many chronic diseases and deeper knowledge about obesity-inflammation interrelationships in adolescent populations, where interaction biases by any ongoing inflammatory pathology that could be present in an adult disease-free population are minimized, is of special interest.

1.1   Overweight and obesity in Spanish adolescents

Overweight and obesity prevalences in the adolescent Spanish population are increasing (Moreno et al. 2000, Moreno et al. 2002). At European level, Spain has one of the highest adolescent obesity prevalences, and is experiencing alarmingly increasing rates of changes.

A simple definition of overweight and obesity is an excess of body fat, or in a more neutral term, adiposity. Adiposity is the amount of body fat expressed either as the absolute fat mass (kg) or as the percentage of total body mass.

Reliable measurements of body fatness are necessary in epidemiological, clinical and population studies, and in the management of nutrition related diseases during adolescence. Anthropometry is the most widely used method to assess the size, shape and composition of the human body, and it's noninvasive and inexpensive. It reflects both health and nutrition and predicts performance, risk factors and survival (de Onis & Habicht 1996). Excess of adiposity requires a suitable measure of body fat and suitable cut-offs. However, in assessing fatness in adolescents an important distinction needs to be made between childhood and adulthood – children grow in size, so that anthropometric cut-offs for fatness have to be adjusted for age and maturation.

Body mass index (BMI; weight/height^2; also known as Queletet's index) is the parameter most frequently used for the screening of excess body fat because it is easy to determine and it tends to correlate well with body fat. In adults, adiposity is commonly assessed using the body mass index and BMI cut-off values generally accepted for the definition of overweight and obesity in adults are 25 and 30 kg/m2, respectively. International Obesity Task Force (IOTF) have proposed BMI cut-off points for children and adolescents, based on BMI centile curves for each half-year of age, which correspond to the adult BMI values of 25 and 30 kg/m^2 at the age of 18 years (Cole et al. 2000). This standard international definition allows the screening of adolescent overweight and obesity worldwide under the same criterion.

It is well known that obesity is an independent risk factor for ill health, but is also strongly associated with insulin resistance (leading to type 2 diabetes), dyslipidaemia and hypertension. This thesis aims to study the relationship of obesity/overweight with inflammatory markers, as another non-conventional risk factor included in the metabolic syndrome (Yudkin et al. 1999, Das 2001).

1.2   Obesity induced chronic low-grade inflammation

Moderately raised inflammatory markers observed in obesity have led to the view that obesity is characterized by a state of chronic low-grade inflammation (Yudkin et al. 1999, Trayhurn & Woods 2004), also in children and adolescents (Ford et al. 2001, Visser et al. 2001).

Adipose tissue is a rich source of many immune related mediators involved in the inflammatory response. Adipose tissue produces pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and complement factors. The hepatic synthesis of acute phase inflammatory proteins, such as C-reactive protein (CRP), ceruloplasmin and complement factors C3 and C4, are in turn under control of these cytokines, and therefore, adipose tissue is a noteworthy factor of raised circulating concentrations of both cytokines and proteins (Trayhurn & Woods 2004).

An important question to answer is how adiposity regulates inflammation during childhood and adolescent overweight and obesity, in order to increase our knowledge of possible mechanisms in related diseases.

1.3   Inflammation and cardiovascular disease in youth

Atherosclerosis is now widely accepted as a chronic inflammatory disease that is initiated by vascular injury induced by oxidized LDL, reactive oxygen species, infection, etc. (Ross 1999, Hansson 2005). A key feature in the inflammation hypothesis is the recognition that circulating immune cells are recruited to the inflamed vessel through the interaction with adhesion molecules and chemokines. We cannot study *in vivo* inflammatory processes in vessels of large groups of healthy young volunteers, but in a simple blood analysis, we can assess immune cells, receptors and inflammatory proteins relatively easily.

Although patients with CVD typically become symptomatic after the age of 40 years, necropsy studies have demonstrated that atherosclerotic changes in the vessel wall begin early in life (Berenson et al. 1992, McGill et al. 2000). Ultrasonography is used in research for the noninvasive detection of atherosclerotic changes in the arterial wall. With this method, CRP has been associated with early arterial changes in children and adolescents (Jarvisalo et al. 2002), supporting the hypothesis that inflammation plays a role in the pathogenesis of early atherosclerosis. There is also increasing evidence to show that chronic subclinical inflammation is associated with metabolic dysfunction which links causally to insulin resistance and the metabolic syndrome (Hotamisligil 2003). Because CVD often has origins at an early age and because several risk factors for CVD track from childhood to adulthood, understanding the distribution and implications of such risk factors among adolescents is of considerable interest for the prevention of future diseases while there is still time to reverse the damage.

1.4 Acute-phase proteins and cytokines

Many markers of inflammation exist, and in this thesis CRP, ceruloplasmin, complement factors C3 and C4, and pro-inflammatory cytokines will be studied.

CRP concentrations are easily, accurately and fairly inexpensively measured in blood. It is an acute phase reactant, produced in the liver and it belongs to the pentraxin family of proteins. This protein is a very sensitive marker of inflammation, and its concentration increases rapidly (100 fold or more) in response to a wide range of stimuli. Belonging to the innate immune response, high CRP levels have no specificity in differentiating disease entities from one another, but despite its lack of specificity, CRP has now emerged as one of the most powerful predictors of cardiovascular risk. In a direct comparison of a panel of inflammatory and lipid markers in predicting cardiovascular events in adults, CRP could surpass other classical risk markers, including LDL cholesterol (Ridker et al. 2001 and 2003). CRP has been shown to have potential effects at nearly every relevant step of atherogenesis, from the earliest changes at sites of future lesions to the accumulation of lipoprotein particles in the sub-endothelial space and threatened rupture of a mature plaque, to the thrombosis that is often the first manifestation of the disease (Lusis 2000).

Complement factors C3 and C4 are other inflammatory markers that are analyzed in this thesis. Complement is the most important component of the humoral autoimmune system. It possesses numerous functions related to host defense, plays a role in clearing immune complexes including antibody-coated bacteria and apoptotic cells and forms interfaces between innate and adaptive immunity by recruiting and activating inflammatory cells. These factors are mainly produced by the liver but are also secreted by activated macrophages in inflammation sites and by adipocytes. While complement activation is accepted to play an important role in acute injury to tissue, its role in the chronic inflammatory state seen in atherosclerosis is less clear. Early components of complement were identified in incipient atherosclerotic vascular lesions almost 20 years ago (Hansson et al. 1984), and in recent years there is increasing evidence that complement activation is involved in the generation of the spontaneous atherosclerotic lesion and may be an initiating factor in lesion formation (Torzewski et al. 1997).

Ceruloplasmin is another protein produced in the liver during the acute phase response. It is rather stable with no more than a 2-fold increase during infection. It is a major plasma protein and functions as a copper transporter that is able to couple and transport 90-95% of serum copper. This protein has also antioxidant functions which can prove beneficial in several pathological conditions, however, ceruloplasmin is not completely harmless because high levels of this protein are associated with atherosclerosis (Engström et al. 2004, Giurgea et al. 2005). Despite these findings the physiological roles of ceruloplasmin are far from understood, and almost not at all studied in healthy asymptomatic young populations.

The main way for the immune cells to communicate with each other and with other cells is regulated by small proteins called interleukins or cytokines. Cytokines are produced mainly by cells within the immune system, e.g. monocytes, macrophages, lymphocytes and T-cells (Th1- and Th 2-cells), but also by other cells, e.g. mast cells, fibroblasts, endothelial cells, neurons and also by adipocytes. Cytokines interact in a complicated way, inducing or inhibiting the production and effects of each other. Sometimes, especially in inflammation, cytokines appear in the circulation most often in order to regulate the gene expression in a specific organ or cell type. This is the case when IL-1, IL-6 and TNF-α induce the hepatic synthesis of acute phase proteins.

1.5　　　Anti-inflammatory effects of physical exercise

It is well established that aerobic exercise is cardioprotective and that being overweight increases the risk of CVD. Given that low-grade inflammation may be involved in obesity-induced chronic diseases as CVD, and given the widespread claims and recommendations that exercise is cardioprotective, one hypothesis could be that good fitness has an anti-inflammatory action and thereby protects against chronic disorders associated with low-grade systemic inflammation. However, because low fitness and increased adiposity often occur in combination, masking their independent effects, it is unclear whether lower fitness or higher body fatness exerts a greater influence on inflammatory risk markers. A healthier immunological profile in subjects with higher physical activity and healthier fitness could simply be due to a generally leaner body composition in active subjects.

In adults, several larger cross-sectional studies have found that physical activity or fitness are associated with lower levels of low-grade inflammation (most studied is CRP) (Geffken et al. 2001, Abramson & Vaccarino 2002, Church et al. 2002, Pischon et al. 2003, Pitsavos et al. 2003, Reuben et al. 2003, Rothenbacher et al. 2003, Albert et al. 2004, Aronson et al. 2004). However, this association has not been confirmed in all studies, especially after adjusting for BMI (Rawson et al. 2003, Verdaet et al. 2004).

Several interesting studies have also been made in children and adolescents assessing this complex relationship between fatness, fitness and inflammation (Cook et al. 2000, Nemet et al. 2003, Halle et al. 2004, Kelly et al. 2004) but possible direct anti-inflammatory effects of a higher fitness in young healthy subjects seem to be less apparent, and more studies are needed to clarify these relationships.

2 AIMS

The overall purpose of this thesis was to increase our knowledge of low-grade inflammatory status in adolescents, and identify determinants such as overweight and fitness; and through this knowledge better understand early stages of future chronic diseases and eventually contribute to improve the health status of the next generation's adult population.

The specific aims of the studies were as follows:

- To present reference values for BMI, sum of skinfolds and body fat percentage in adolescence;

- To determine if there is an association between overweight and a state of chronic, low-grade inflammation in adolescence;

- To examine the relationships between several acute phase proteins and body fat estimates and distribution;

- To analyze interactions of fitness and fatness on low-grade inflammation across different levels of adiposity and fitness in adolescence.

3 SUBJECTS AND METHODS

3.1 Setting and design (AVENA study)

The Spanish multicenter study AVENA (Alimentación y Valoración del Estado Nutricional en Adolescentes: Food and Assessment of the Nutritional Status of Adolescents) was designed to evaluate the nutritional status of a geographically representative sample of Spanish adolescents in order to identify risk factors for chronic diseases in adulthood. Due to its importance and adequate development, the study was financed by the Spanish Ministry of Health, through FIS (Fondo de Investigación Sanitaria) contract nr 00/0015.

3.2 Methodology for standardized studies in adolescents

An important objective of the AVENA study was also to develop a validated methodology to evaluate the health and nutritional status of the adolescent population, for standardization between national studies and also for comparison of studies world-wide. With this purpose, the AVENA methodology is now being used in two ongoing studies to assess adolescent nutritional status and several other studies on adolescents are being planned with the same methodology.

The European Union funded project called "Healthy Lifestyle in Europe by Nutrition in Adolescence" (HELENA) (contract number: FOOD-CT-2005-007034), was designed by AVENA partners and its cross-sectional methodology is improved but almost intact. The HELENA study includes 3000 adolescents from 10 European countries, including Sweden. The study started in 2005 and will go on to 2008. Also in South America, the AVENA methodology is, and is planned to be, used in several cross-sectional nutritional studies in adolescents. In Mexico, the Universidad Autónoma del Estado de Mexico in Toluca City is replicating the AVENA methodology in 1412 adolescents, the project was initiated in February 2005 and will be concluded in November 2006. The same will most probably be replicated in other Northern and Southern Mexican Universities in order to obtain National Mexican Data. Researchers in Argentina, Venezuela, Chile and Guatemala are designing similar studies that will be using the same methodology.

3.2.1 Sampling procedure and study population

The AVENA study was performed on Spanish adolescents aged 13–18 years from 5 Spanish cities (Granada, Madrid, Murcia, Santander and Zaragoza), rather homogeneously distributed according to Spain's geography (Figure 1), during years 2000-2002.

Figure 1. Map of Spain with cities participating in the AVENA study.

The population was selected by multistep, simple random sampling, taking into account first the location and then by random assignment of the schools within each city. The sample size was stratified by age and sex. The socio-economic variable was considered to be associated to geographical location and type of school. As the selection of schools was done by random selection and fulfilling quota, this variable was also considered to be randomly assigned. To calculate the number of adolescents to be included in the study in order to guarantee a representative sample of the whole country, we selected the variable with the greatest variance for this age group from the data published in the literature at the time the study was planned; that was BMI (Moreno et al. 1997). The sampling was determined for the distribution of this variable; the CI was established at 95% with an error of $\pm$ 0.25. The established number of subjects was 2100. The total number of subjects was uniformly distributed in the five cities and proportionally distributed by sex and age group (13, 14, 15, 16, 17-18 years). The sample was oversized in order to prevent loss of information and because technically it was necessary to do the fieldwork in complete classrooms. After finishing the field study, the subjects who did not fulfill the inclusion criteria were excluded.

Exclusion criteria were: type 2 diabetes, pregnancy, alcohol or drug abuse and nondirectly-related nutritional medical conditions. Finally, the sample was adjusted by a weight factor in order to balance the sample in accordance to the distribution of the Spanish population and to guarantee the representativeness of each of the sex and age groups.

Blood samples were obtained in a randomly selected sub-sample of 500 subjects. This subsample was equal to the full AVENA sample according to BMI, age and sex distribution (Ruiz et al. 2006). Before blood sampling, each subject was evaluated and anamnesis data were registered and reviewed to judge the adolescent's medical status. Only healthy adolescents were included in studies with immunological data. Thus, subjects presenting chronic diseases thought to have possible effect on immune function or taking any medications with known immunological effects were excluded from the study (e.g., asthma). Subjects with any acute medical conditions, such as minor infections (upper respiratory illness) were also excluded. After finishing the fieldwork, the subjects who did not fulfill the inclusion criteria were excluded.

3.2.2   Ethics

A detailed verbal description of the nature and purpose of the study was given to the adolescents and their teachers. This information was also sent to their parents/guardians by letter and written consent was requested from both parent/guardians and the adolescent. Only on receipt of their written consent, were the subjects considered for inclusion in the study.

The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), following the EEC Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law which regulates clinical research in humans (Royal Decree11 561/1993 regarding clinical trials). The study protocol was approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

3.3   AVENA methodology

In the AVENA study a multidisciplinary methodology was assessed, with experts from 5 centers (one from each of the 5 cities) responsible for each research area; in Madrid, the Consejo Superior de Investigaciones Científicas (CSIC) was responsible for the coordination and overall anamnesis, haematological, immunological and psychological assessments; the university of Zaragoza was responsible for body composition assessment; the university of Granada was responsible for biochemical, fitness and physical activity assessments; the university of Cantabria (Santander) was responsible for genotyping assessment, and the university of Murcia for the dietary records.

All methods used in the AVENA study are summarized in Tables 1 and 2, although only gender, age, pubertal maturation, body composition, fitness and inflammatory markers are included in this thesis.

Blood variables analyzed in the subgroup with blood samples are summarized in Table 2, although in this thesis only serum inflammatory proteins and in vitro production of cytokines IL-6 and TNF- α are included.

Table 1. Variables studied within the AVENA study

Study variables	Methods
Personal background and environmental conditions	
Gender, age, ethnicity, socioeconomical status, anamnesis, family history of diseases, gestation time, birth weight, breast feeding.	Parental questionnaire, clinical examination, interviews.
Environmental data (family composition and habits)	Parental questionnaire
Body composition and maturation	
Height, weight, skinfolds, circumferences	Anthropometrical assessment
Body fat*	Dual-energy X-ray absorptiometry
Pubertal maturity and age of menarche	Tanner stage, clinical examination, interviews.
Physical fitness	
Muscle strength (grip, arm and shoulder, legs) agility and flexibility	Eurofit battery
Cardiorespiratory fitness (VO$_2$max estimation)	20 m Shuttle run test
Physical activity	
Physical activity at weekday, weekend and summer	Questionnaire
Personal approach to physical activity	Questionnaire
Dietary assessment	
Dietary recall	24h dietary recall
Food frequency	Food Frequency Questionnaire
Food diary	7-day food diaries
Food habits and nutrition knowledge	Questionnaire
Psychology	
Screening for eating disorders	5 SCOFF questions
Behavioral, psychological traits in eating disorders	Questionnaire (Eating Disorders Inventory)
Intellectual performance (verbal aptitude, logical reasoning, mathematical skills)	Questionnaire (Test de Aptitudes Escolares-TEA)

* Assessed in a subgroup of 238 subjects (AVENA-Zaragoza city only)

Table 2. Blood variables assessed in the AVENA subgroup with blood sampling

Analysed blood variables	Methods
Hematology and biochemistry	
White and red blood cell counts	Standard cell counter
Lipid profile: Triacylglycerol, total-, HDL-, VLDL-, LDL-cholesterol	Standard enzymatic-colorimetric analyzer
Lipoproteins ApoA1, ApoB, lp(a)	Immunonephelometry
Glucose, prealbumin, iron, total protein, creatinine, calcium, phosphorus, urea, ureic acid	Standard automatic analyzer and nephelometry
Immunological parameters	
Innate immunity	
CRP, C3, C4, ceruloplasmin	Standard turbidimetry analysis
Natural Killer cells ($CD56^+CD16^+$)	Immunophenotyping
Phagocytosis and oxidative burst*	PhagoTest®, Burst test® measured by flow cytometry
Cell-mediated immunity	
T-lymphocyte subpopulations ($CD2^+$, $CD3^+$, $CD4^+$, $CD8^+$)	Immunophenotyping
In vitro production of cytokines by isolated, stimulated white blood cells (IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10)	Cell culture of PBMC, cytokine detection in supernatant by CBA® and flow cytometry
In vivo cell mediated immunity**	Multitest® intradermal skin test
Humoral immunity	
Serum immunoglobulins G, A, M	Standard turbidimetry analysis
B-lymphocytes ($CD19^+$)	Immunophenotyping
Genotype polymorphisms	
APOE, APOC3, PPAR-γ	Genotyping

* Assessed in a subgroup of 90 subjects (AVENA-Madrid city only)

** Assessed in a subgroup of 79 subjects (AVENA-Madrid city only)

3.3.1 Anthropometry

All anthropometrical measurements were made with subjects barefoot and in their underwear. Body mass index (BMI) was calculated as body weight (kg), divided by height (m) squared. Body weight was measured to the nearest 0.05 kg using a standard beam balance. Skinfold thicknesses were measured at the left side of the body to the nearest 0.2 mm with Holtain skinfold calipers, at the following sites: 1) triceps, halfway between the acromion process and the olecranon process; 2) biceps, at the same level as the triceps skinfold, directly above the centre of the cubital fossa; 3) subscapular, about 20 mm below the tip of the scapula, at an angle of 45° to the lateral side of the body; 4) suprailiac, about 20 mm above the iliac crest and 20 mm towards the medial line; 5) thigh, in the midline of the anterior aspect of the thigh, midway between the inguinal crease and the proximal border of the patella; 6) calf, at the level of maximum calf circumference, on the medial aspect of the calf. Circumferences were measured in cm with an inelastic tape to the nearest 1 mm. In general, for these measurements, the subject was in a standing position. To measure the waist circumference, the tape was applied horizontally midway between the lowest rib margin and the iliac crest, at the end of gentle expiration. The hip circumference measurement was taken at the point yielding the maximum circumference over the buttocks, with the tape held in a horizontal plane. The complete set of anthropometric measurements was performed three times, but not consecutively; we measured all the anthropometric variables in order, and then we repeated the same measurements two more times.

Harmonization and standardization of anthropometric measurements within the AVENA multicenter study was strictly controlled during the AVENA pilot study. Intra-observer reliability for skinfold thicknesses was higher than 95% for almost all the cases; inter-observer reliability for skinfold thickness ranged from 83.05 for biceps skinfold to 96.38 for the calf skinfold (Moreno et al. 2003).

As an index of total adiposity we calculated the sum of the six measured skinfold thicknesses. Body fat percentage was calculated by the formulae described by Slaughter et al. (1988) because this equation showed the best agreement with total body fat percentage measured by dual energy X-ray absorptiometry (DXA) in a subsample of 238 AVENA subjects from Zaragoza (Rodriguez et al. 2005).

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Overweight and obesity status in adolescents were in this thesis established using the international standards, based on BMI, age and gender (Cole et al. 2000).

Central body fat accumulation was described as waist circumference alone and waist-to-hip circumference ratio (WHR), together with the percentage of truncal (subscapular + suprailiac) to total sum of 4 skinfolds (biceps, triceps, subscapular and suprailiac).

During anthropometrical measurements, pubertal maturity was classified according to one of the five stages defined by Tanner & Whitehouse (1976). The standard staging of pubertal maturity describes breast and pubic hair development in girls and genital and pubic hair development in boys.

3.3.2   Cardiorespiratory fitness

Maximum aerobic capacity was assessed by an indirect incremental maximum 20 m shuttle run test (Léger et al. 1984). This field test is a good option to evaluate a large number of subjects at the same time. Subjects were instructed to abstain from strenuous exercise for 48 h before the test. Running pace was determined by audio signals emitted from a pre-recorded cassette tape; the initial velocity was 8.5 km·h-1, which was increased by 0.5 km·h-1 per minute (i.e., per stage). Subjects were instructed to run in a straight line, to pivot upon completing a shuttle, and to pace themselves in accordance with the audio signals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on two consecutive occasions. A constant level of encouragement was given to participants throughout the test. Scores were recorded as the number of stages completed.

The equations by Léger et al. (1988) were used to estimate the maximum oxygen consumption (r=0.7 for 8-19 years-old children and adolescents). The formula to obtain VO_2max estimates from the results of the 20 m shuttle run test is: $VO_2max = 31.025 + 3.238 \times S - 3.248 \times A + 0.1536 \times SA$, where S is the final speed ($S = 8 + 0.5 \times$ last stage completed) and A is age in years. The reliability and validity of this test for determining the VO_2max in children and adolescents have been widely documented (Van Mechlen et al. 1986, Léger et al. 1988, Liu et al. 1992).

3.3.3 Blood samples

Fasting venopuncture blood samples were collected at the schools between 8:00 and 9:30 a.m. A maximum of 20ml of blood was withdrawn, and three tubes were filled when possible (6ml in heparin, 3ml in EDTA and 9ml were allowed to clot in SST). Fasting conditions for 8h and abstention from physical exercise during the previous day were confirmed at the time of the extraction.

Blood for serum was received in our laboratory a maximum of 2h after clotting and centrifuged for 15 minutes at 3500rpm. The serum was divided into aliquots, and 0.5ml was frozen and stored at $-80ºC$ until withdrawn for analysis of acute phase proteins.

Highly sensitive CRP (hs-CRP) ceruloplasmin, C3 and C4, were measured in serum by immunoturbidimetry (AU2700 biochemistry analyzer; Olympus, Rungis, France). The coefficients of variation, %CV (inter-assay precision), were less than 2% for all proteins (1.90% for CRP, 2.00% for ceruloplasmin, 1.39% for C3 and 1.19% for C4). Detection limits (sensitivity) for the analyses were 0.007 mg/L for CRP, 0.04 g/L for ceruloplasmin, 0.01 g/L for C3 and 0.002 g/L for C4. Single-point measurement was performed for all analyses. All samples were analyzed at the same time, in the end of the study, to minimize systematic variation.

In vitro production of cytokines by isolated, stimulated peripheral blood mononuclear cells (PBMC) was assessed in cultured mitogen-stimulated cells. The 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 1ml of cell suspension was incubated per well with mitogens, phytohemagglutinin (PHA 3.5 µl/ml) and lipopolysaccharide (LPS 1.5 µl/ml) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), in 24-well plates for 48 h, at 37ºC and 5% CO^2. Following incubation the cells were removed by centrifugation and supernatant stored at $-80ºC$ until withdrawn for analysis.

Cytokine (interleukin [IL-6] and tumor necrosis factor α [TNF-α]) content of the supernatant was analyzed using the Human Th1/Th2 cytokine CBA II kit (BD Biosciences Pharmingen, San Diego, CA), and detected by flow cytometry. Detection limits for IL-6 was 3.0 pg/mL and for TNF-α 2.8 pg/mL. Intra-assay precision was less than 3% and inter-assay precision ranged from 3 to 7% for IL-6 and 3 to 6% for TNF-α, depending on the level of cytokine in the sample tested; the lower the concentration, the higher the %CV.

Single-point measurement was performed and cytokine detection in all samples was analyzed at the same time, in the end of the study, to minimize systematic variation.

3.4 Statistical methods

Statistical analyses were performed using SPSS versions 11.5 to 13.0 for Windows XP. In all analyses, statistical significance level was set at $\alpha = 0.05$.

In study I, gender differences in each age group were assessed by Mann–Whitney U test. Differences between age groups in each gender by Kruskal–Wallis test.

Centile values were calculated and curves were fitted to the data using Cole's LMS method (Cole et al. 1998). Briefly, this assumes that the data can be transformed to normality by a suitable power transformation (L), and the distribution is then summarized by the median (M) and coefficient of variation (S). The values of L, M and S are constrained to change smoothly with age, and the fitted values can be used to construct any required centile curves. (Study I).

CRP, IL-6 and TNF-α were transformed logarithmically and C3, C4 and ceruloplasmin were square rooted to stabilize variability and to achieve normality in the residuals in Studies II-IV. Transformed data was used for statistical analysis although raw values are presented in tables and figures. Subjects with overweight and obesity were studied together to obtain a larger comparison group for stronger statistical relevance. Basic descriptive statistics of inflammatory markers were presented as means ± S.D for each gender and weight status group.

Student's t-test was used for comparison between the non-overweight and overweight/obesity groups (Study II), and gender by weight status interactions were analyzed by 2-factor ANOVA (Study III). Analyses of partial correlation coefficients, adjusted for age and Tanner stages between protein concentrations and anthropometric parameters and indices of body fat and distribution were calculated in study III. Tanner stages were entered as dummy variables.

Odds ratios for having clinically significant high values of inflammatory proteins across overweight and fitness groups were calculated by 2-way contingency tables (Study IV). χ^2-test and Fisher's exact test was used for levels of significance (bilateral).

To examine the independent association of fatness and fitness with inflammatory proteins in Study IV, multiple linear regression analyses were made. For each model, inflammatory proteins were entered as the dependent variable, whereas fitness, BMI, and age were entered as predictors (independent variables). Residual analyses to test the validity of the regression model assumptions were performed for all the regression models.

4   RESULTS AND GENERAL DISCUSSION

4.1   Descriptive data

The participation rate was over 80% in the five cities, but no further gender or socio-economical study of the non-participants was done.

4.1.1   Gender, age and pubertal maturation

The final number of subjects included in the AVENA Study was 2859 adolescents. 2160 adolescents had a complete set of anthropometric measurements and were therefore included in Study I. The number of adolescents in each age group, and gender distribution is shown in Table 3.

Table 3. Age and gender distribution of the AVENA sample.

Age-groups	%	n	Males (n)	Females (n)	Males (%)
13	19%	417	223	194	53%
14	20%	431	215	216	50%
15	21%	448	238	210	53%
16	19%	407	209	198	51%
17-18	21%	457	224	233	49%
Total sample	100%	2160	1109	1051	51%

The distribution of Tanner stages in males was 4.87% in stage II, 13.98% in stage III, 36.97% in stage IV and 44.18% in stage V. In females, the corresponding distribution was 0.76%, 10.75%, 49.19% and 39.30%. For females, the self-reported age of menarche ranged from 9 to 15 years of age. The distribution of age of menarche was: 9 years (1.6%), 10 years (2.9%), 11 years (20.9%), 12 years (34.7%), 13 years (27.4%), 14 years (11.3%), and 15 years (1%).

Blood samples for immunological study were obtained in a randomly selected sub-sample. This subsample was equal to the larger AVENA sample according to BMI, age and gender distribution, as seen in Table 4.

Table 4. Comparisons of body mass index between sub-group in which blood samples for immunological analysis were obtained (blood group) and the group without blood samples (non blood group).

Age groups (years)	Body Mass Index		P
	Blood group	Non Blood group	
Males			
13	20.3 ± 3.1	20.7 ± 4.0	0.61
14	22.1 ± 4.0	21.4 ± 3.5	0.16
15	22.4 ± 3.9	21.9 ± 3.5	0.38
16	22.1 ± 3.3	21.7 ± 3.1	0.43
17-18	23.6 ± 4.3	22.7 ± 3.5	0.26
Females			
13	21.5 ± 3.8	21.5 ± 3.7	0.93
14	21.7 ± 4.2	21.2 ± 3.5	0.47
15	21.5 ± 3.3	21.4 ± 3.0	0.96
16	22.3 ± 3.0	21.5 ± 3.1	0.06
17-18	21.9 ± 2.9	21.7 ± 3.2	0.64

493 adolescents with CRP, IL-6 and TNF-α data together with BMI for overweight classification were included in Study II. 472 adolescents with a complete set of anthropometrical data and CRP, C3, C4 and ceruloplasmin concentrations were included in Study III. From these, 444 adolescents with BMI for overweight classification and fitness data (VO_2max estimate) were included in Study IV.

4.1.2　Basic characteristics

In 2000-2002 most Spanish adolescents were Caucasians, but limited groups of other races and ethnic populations were included in the study, as whole classrooms were studied. Ethnicities of the study population were 2.5% Latin-Americans, 0.5% Asians, 0.2% Africans and 0.2% gypsies. According to socio-economical status (by means of the educational level and the type of occupation of the father) 5.1% adolescents were from low socio-economic class, 17.4% from medium-low, 25.0% from medium, 17.3% from medium-high, and 4.9% from high socioeconomic status. Sub analysis stratified by socio-economic status or race was not possible as the groups were too small or did not have any effect on the analysis of the data in Studies II-IV, and was not taken into account.

4.1.3   Body composition and overweight prevalences (Study I)

The studied sample was designed to be representative of the real situation in Spain, and our results show an alarming overweight + obesity prevalence of 25.69% (95% CI: 23.22, 28.16) and obesity prevalence of 5.68% (95% CI: 4.37, 6.99) in males while the figures in females are a little lower: overweight (including obesity) prevalence of 19.13% (95% CI: 16.84, 21.42), and obesity prevalence of 3.08% (95% CI: 2.08, 4.08). (Moreno et al. 2005). We have used the International Obesity Task Force reference standard to define weight status (Cole et al. 2000), since this tracks logically through childhood, adolescence and into adulthood. More importantly, this standard international definition allows the screening of adolescent overweight and obesity worldwide under the same criterion.

In Study I, means and age-related reference centiles for body fat composition indices for Spanish adolescents 13–18 years are presented. These will provide baseline data for Spain from which to track future trends and to assess the impact of intervention studies in this population. Mean values of sum of 6 skinfolds and body fat % in each age group were significantly higher in females than in males. In males, age was significantly associated with higher BMI, sum of 6 skinfolds and body fat %; however, in females, the effect was only significant for body fat %. Among age-groups, mean values of BMI ranged from 20.61 to 22.91 kg/m^2 in males, and from 21.33 to 21.72 kg/m^2 in females. Mean values of body fat % ranged in males from 18.28 to 20.79 and in females between 24.89 and 26.30.

Smoothed LMS (age- and gender-specific) curves for the 5th, 10th, 25th, 50th, 75th, 90th and 95th percentiles of BMI, sum of 6 skinfolds and body fat % are presented in Study I. In boys, BMI 50th percentile ranged from 19.80 to 22.33 kg/m^2; and in girls ranged from 20.61 to 21.17 kg/m^2. 50th percentile of body fat % ranged from 16.10 to 18.18 in males; and from 23.89 to 25.36 in girls. The percentile distribution was more disperse towards higher sum of 6 skinfolds and body fat % values in males when compared with females.

Determination of fat mass is of considerable interest in the evaluation of nutritional status. Both over-and under-nutrition contribute to increased mortality and morbidity. Ideally this evaluation should be made using direct measures such as DXA or underwater weighing, but these techniques require sophisticated laboratory settings and are limited because of the required performance of the participants. In clinical settings and epidemiological studies, other measurements are used, including skinfold thickness measures and bioelectrical impedance. The most common estimate of excess or deficit in body adiposity is, however, the body mass index (BMI). No measure optimally describes obesity in all age-sex-race groups because of differences in bone density, muscle and adipose tissue. The BMI is easy to measure and calculate, but it includes measures of bone mass and fat free mass, as well as fat mass. Although there are well known limitations regarding the use of BMI (Wang 2004), statures and weights of children and adolescents are easily and reliably obtained in a wide variety of clinical settings.

Body fat % was measured by DXA (Dual-energy X-ray absorptiometry) in a sub-group of 280 adolescents from Zaragoza, (Rodriguez et al. 2004), and subjects were classified into three groups: normal-weight, overweight and obese defined by IOTF cut off values (Cole et al. 2000), further, the 85th percentile of the body fat % distribution in each gender was considered as the cut off point for excess adiposity. In total, 94% of males and 84% of females with high adiposity were correctly identified by BMI (sensitivity), and 88% of males and 93% of females without high adiposity were also correctly classified (specificity). According to this study, BMI cut-offs seemed to be a good criterion for the screening of excess body fat in adolescents; however an important percentage of subjects classified as overweight or obese did not really have excess adiposity. One interesting point of the results of Study I is the finding that the increase in BMI in this population is mainly caused by the increase in the upper levels of the BMI distribution, pointing that those susceptible individuals, are more and more obese.

The presented percentile values will help us to classify adolescents for future comparison with a well-established reference population, and to estimate the proportion of adolescents with high or low adiposity levels.

4.1.4 Cardiorespiratory fitness (Study IV)

Cardiorespiratory fitness data are included in Study IV in this thesis, for the study of possible effects of a higher fitness on previously found relationships between body fat and low-grade inflammation from Studies II-III. A detailed descriptive study of fitness status in the 2858 Spanish adolescents studied within the AVENA study, representing reference values in Spanish adolescence, is the base for another thesis and can be consulted in several papers (Ortega et al. 2005, Ortega et al. 2006).

As background information, poor cardiorespiratory fitness is found in our sample and thus in the Spanish adolescent population. We have compared these results with data from other countries, and the fitness status was shown to be lower in Spaniards. As much as 20% of Spanish adolescents are estimated to have a level of physical fitness that could be indicative of future CVD. (Ortega et al. 2005).

Age-adjusted correlations between % body fat and VO_2max estimate was, as expected, high, reflecting the fact that a fit adolescent has less body fat than its sedentary peer. Mean values of VO_2max in the 444 adolescents included in Study IV was 49.16 ± 9.56 ml/kg/min in males and 42.35 ± 8.10 ml/kg/min in females (Gender difference: $P<0.0001$).

4.1.5 Inflammation markers (Study II-IV)

CRP concentration ranged from 0.04 to 47.37 mg/L (median, 0.63 mg/L). CRP concentrations >10mg/L may reflect an acute phase response to infectious disease or disorders characterized by acute inflammation, and to minimize the confounder of an ongoing infection, 9 subjects (6 non-overweight and 3 overweight) with CRP >10mg/L were excluded from all further analyses. Mean values ± SD of the studied inflammatory parameters are shown in Table 5.

Table 5. Mean values ± SD of the studied parameters for the entire sample and genders separately.

	Males	Females	All
CRP (mg/L)*			
Mean ± SD	1.32 ± 1.60	0.93 ± 1.08	1.13 ± 1.38
95% CI	1.13 – 1.52	0.80 – 1.07	1.02 – 1.26
Ceruloplasmin (g/L)*			
Mean ± SD	0.20 ± 0.05	0.22 ± 0.05	0.21 ± 0.05
95% CI	0.20 – 0.21	0.21 – 0.22	0.21 – 0.21
C3 (g/L)*			
Mean ± SD	1.37 ± 0.25	1.33 ± 0.23	1.36 ± 0.24
95% CI	1.35 – 1.41	1.31 – 1.37	1.34 – 1.38
C4 (g/L)			
Mean ± SD	0.27 ± 0.09	0.26 ± 0.10	0.27 ± 0.10
95% CI	0.26 – 0.28	0.25 – 0.28	0.26 – 0.28
IL-6 (mg/L)			
Mean ± SD	33840 ± 21485	35538 ± 21014	34664 ± 21249
95% CI	30953 – 36729	32630 – 38446	32622 – 36708
TNF-a (pg/L)			
Mean ± SD	2394 ± 2156	2224 ± 2270	2312 ± 2211
95% CI	2105 – 2683	1911 – 2538	2100 – 2524

Gender differences were found in CRP ($p<0.005$) and C3 ($p<0.05$), with higher values in males, and for ceruloplasmin ($p<0.01$), with higher values in females.

The American Heart Association and the Centers for Disease Control and Prevention in the USA issued a joint statement regarding the public health and clinical utility of new information regarding markers of inflammation and their relationship with CVD (Pearson et al. 2003). The statement confirmed that CRP is the best and most clinically useful of the markers of inflammation currently available, with the following cut-off points for assessing CVD risk using CRP: low risk (CRP < 1.0 mg/L), average risk (1.0–3.0 mg/L), and high risk (>3.0 mg/L). However, these cut-off points are for adults and no references are stated for adolescents or children. Therefore, and due to the lack of universal clinical cut-off points for the definition of risk values of the other studied acute phase proteins, the highest age-adjusted quartile of CRP, ceruloplasmin, C3 and C4 concentrations were considered high values in Study IV.

Although throughout this thesis I suggest that high values could promote future disease, it is important to stress that the serum values of inflammatory proteins shown in these studies are always within normal ranges, which makes us consider the whole sample as healthy subjects. However, the role of chronically high values may be clinically significant in the long term.

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 Study II and III. The values are not homogenously distributed and their variability is large. When we evaluate *in vitro* measurement of cell function in isolated blood mononuclear cells, it must be borne in mind that the majority of immune cells are not in the bloodstream, only 2% of the total lymphocytes are circulating at any given time (Westermann & Pabst 1990). Isolation of cells from the bloodstream allows us a precise control over the number and types of cells being studied. In our case, mononuclear cells in a concentration of one million cells per mL of culture medium are cultured under controlled conditions, and exact concentrations of cytokines produced by these cells are then measured. Isolation removes the cells from the other cell types and blood constituents (e.g. hormones, cytokines or adipokines as leptin) that they would normally be in contact with and this may alter *in-vitro* responses compared with those that the cells might undergo *in vivo*. This places a limit on extrapolation of findings in cell culture to the whole body situation. However, this is to date the best assessment of the function of immune cells in the blood stream, and a lot of research is still needed to understand the complexity of the cell to cell signalling by cytokines.

4.2   Obesity-inflammation interrelationships (Study II-IV)

In Studies II-IV, obesity-inflammation interrelationships are explored. In Table 6, means ± SD of serum concentrations of inflammatory proteins and *in vitro* production of IL-6 and TNF-α among Spanish adolescents, divided in non-overweight and overweight/obesity are shown. Significantly higher inflammatory markers in the overweight group characterize this apparently healthy young overweight population by a state of chronic low-grade inflammation. Associations between inflammatory proteins, pro-inflammatory cytokines and adiposity measurements were examined in Study III and we found that acute phase inflammatory proteins CRP, C3 and C4 were all positively correlated with most measurements of body fat while ceruloplasmin only correlated with measures of body fat in females. There could be several causes for the association between overweight and body fat with chronic low-grade inflammation, but the mechanisms are unfortunately not entirely understood.

One direct mechanism could be that adipose tissue is a direct source of pro-inflammatory cytokines such as IL-6 and TNF-α (Trayhurn & Woods 2004), which in turn induce hepatic synthesis of acute-phase proteins such as CRP, C3, C4 and ceruloplasmin (Ramadori & Christ 1999), In Study II we report small but not significant increases in the production of IL-6 and TNF-α by white blood cells in overweight adolescents, however, no associations were observed with any measures of body fat. It must be borne in mind that this study included the determination of IL-6 and TNF-α production by *in vitro* stimulated white blood cells, which is just one source of the overall production in the body. Adipose tissue, and the visceral depot in particular, is a major contributor of plasma IL-6, explaining approximately 33% of plasma IL-6 concentrations (Fried et al. 1998, Mohamed-Ali et al. 1997). On explanation could thus be that cytokines produced locally by adipocytes and not by circulating white blood cells induce an acute phase response. Direct assessment of serum pro-inflammatory cytokines is needed in future studies to further confirm this hypothesis. One limitation is, however, the low serum cytokine concentrations in healthy young populations, which fall below the assay's sensitivity in a high number of cases.

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Table 6. Serum concentrations of inflammatory proteins and *in vitro* production of IL-6 and TNF-α among Spanish adolescents, divided in non-overweight and overweight/obesity.

	Non- Overweight	Overweight and Obesity	P
CRP (mg/L)			
Males	1.17 ± 1.62	1.68 ± 1.55	0.022
Females	0.83 ± 0.86	1.33 ± 1.47	0.003
All	0.99 ± 1.30	1.55 ± 1.52	<0.0005
Ceruloplasmin (g/L)			
Males	0.20 ± 0.04	0.21 ± 0.05	NS
Females	0.21 ± 0.05	0.25 ± 0.05	<0.0005
All	0.21 ± 0.05	0.22 ± 0.05	0.001
C3 (g/L)			
Males	1.32 ± 0.22	1.51 ± 0.28	<0.0005
Females	1.29 ± 0.20	1.46 ± 0.28	<0.0005
All	1.31 ± 0.21	1.50 ± 0.28	<0.0005
C4 (g/L)			
Males	0.25 ± 0.81	0.32 ± 0.10	<0.0005
Females	0.26 ± 0.09	0.31 ± 0.11	0.002
All	0.25 ± 0.09	0.31 ± 0.10	<0.0005
IL-6 (pg/mL)			
Males	32306 ± 19549	37264 ± 25527	NS
Females	35263 ± 21967	39137 ± 19683	NS
All	33825 ± 20844	37971 ± 23403	NS
TNF-α (pg/mL)			
Males	2290 ± 1624	2724 ± 3095	NS
Females	2275 ± 2451	2276 ± 1825	NS
All	2282 ± 2088	2556 ± 2088	NS

P for comparisons between weight status groups (ANOVA). Statistical analysis was made with transformed data (lnCRP, sqrtC3, sqrtC4, sqrtCeruloplasmin, lnIL-6 and lnTNF-α).

Another cause of increased inflammation with overweight could be the associations that have been found between obesity and oxidative stress, which in turn could increase inflammation (Perticone et al. 2001).

Although the general assumption is that inflammation is consequent to excess adiposity, we cannot rule out that obesity could be a result of inflammatory disease (Das 2001).

In adults, it is now well recognized that CRP and BMI are strongly associated (Ford 1999, Yudkin et al. 1999), but this has also been documented in children and adolescents (Cook et al. 2000, Visser et al. 2001, Ford et al. 2001, Vikram et al. 2003, Wu et al. 2003). Our results are in accordance with previous studies of this association.

More interesting are the much less studied complement factors. Associations with body fat were generally stronger for C3 and C4 than for CRP, and most interesting was the finding that C3 was associated, above the other studied inflammatory proteins, to abdominal obesity. We can speculate that the strong relationships between body fat and C3 and C4 found in this study could be explained by an increased production in adipocytes, apart from other complement producing tissues (hepatic cells, fibroblasts, mononuclear cells and endothelial cells) (Niculescu et al. 2004, Trayhurn & Woods 2004). Indeed, both C3 and C4 have been related to obesity in adults (Gabrielsson et al. 2003, Muscari et al. 2005, Onat et al. 2005). Further data supporting the actions of body fat on serum complement factors are findings in anorexic patients, where C3 concentrations are low and normalize with weight gain and on the other hand, obese adults have higher C3 concentrations than controls and the levels decrease after weight loss (Pomeroy et al. 1997, Nova et al. 2004). However, studies of complement factors in younger populations have only been done in pathological conditions (Moreno et al. 1994, Cianflone et al. 2005), and this is probably the first population-based study to show this association in apparently healthy adolescents.

Three of the four acute phase proteins assessed in this study showed clear linear associations with adiposity in both males and females. However, the relationships were different for ceruloplasmin, which showed association to body fat only in females. Similarities should be taken with caution, but of interest is the finding that ceruloplasmin was unrelated to BMI in men with low risk factors, although relations with mortality and CVD were found (Engström et al. 2004). Although inflammation is mediated by all acute phase proteins, their relation to adiposity in healthy adolescents is different, as shown by the results of this thesis, and their different roles need further study.

Although inflammation is a key function within the immune response, inflammatory processes are involved in the pathogenesis of CVD and may also play an important initiating role in the development of the cardiovascular lesion. If moderate chronic elevations of inflammatory proteins are markers of ongoing vascular disease and is predicting future diseases or if inflammatory proteins are merely practical markers of body fat is beyond the possible answers of this study. Whether chronic low-grade inflammation has any direct physiologic or pathologic implication is still unclear, and we can from this study only speculate about possible consequences.

Clinical and scientific data linking inflammation, CRP and atherosclerosis continue to accumulate, and in adults, CRP has shown significant associations with CVD, and is well recognized as a potent CVD risk factor (Ross 1999, Hansson 2005). In pathology studies, CVD 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 (Jarvisalo et al. 2002), supporting the hypothesis that inflammation plays a role also in the pathogenesis of early atherosclerosis. We can thus from our data of raised CRP values, fear that the studied overweight Spanish adolescents could have an increased risk for future CVD.

Components of the complement cascade, which mediate tissue damage have also been identified in early atherosclerotic plaques (Torzewski et al. 1997) and have been shown to co-localize with CRP. Inflammatory cells in these plaques as well as CRP, link complement proteins to local arterial disease (Yasojima et al. 2001). In fact, one of the first physiologic roles identified for CRP was its ability to activate complement. This has *in vitro* been shown to be progressively increased in the presence of free-radical modified cell membranes (Klegeris et al. 2002), which suggests that at sites of tissue inflammation this may be an important mechanism activating complement. More research is needed to clarify the role of the complement system in atherosclerosis, but nevertheless, the deposition of C3 and the activation of complement in arteries could potentially promote atherogenesis (Bhakdi et al. 1999), and the implication of raised values with total and central obesity in adolescents needs further study.

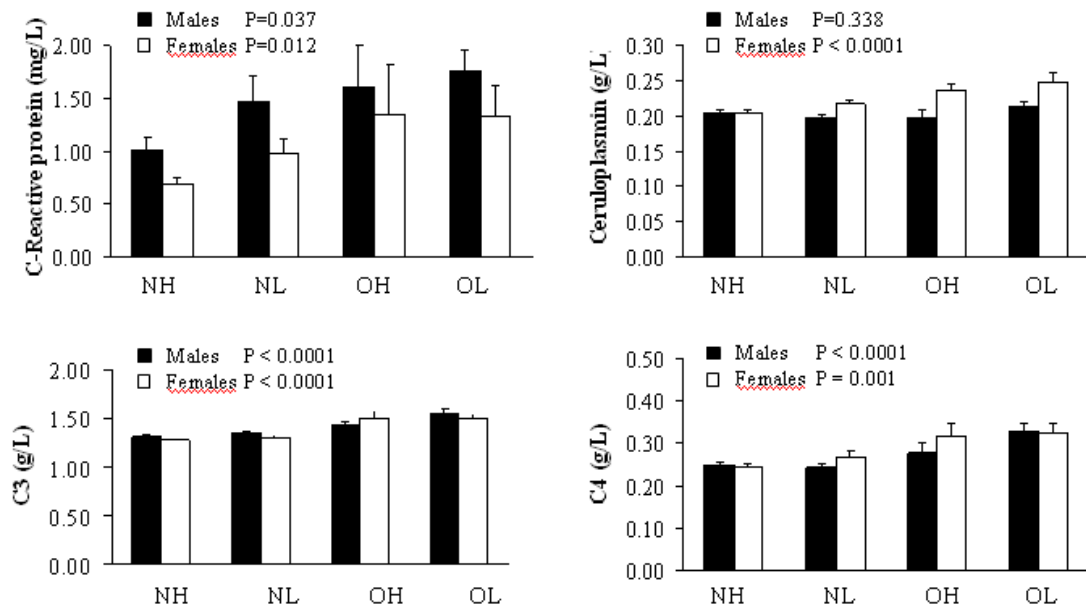## 4.3   Fitness and obesity-inflammation interrelationships (Study IV)

It has been suggested that poor physical fitness during adolescence could be associated with later CVD risk factors (Boreham et al. 2001, Boreham et al. 2002, Hasselstrøm et al. 2002, Janz et al. 2002). However, the mechanisms for the cardiovascular benefits of physical fitness are not completely understood.

Good physical fitness seems to counteract the relationship between obesity and increased cardiovascular risk, and it has even been suggested that obese but fit individuals are at lower risk for cardiovascular risk and all-cause mortality than normal weight, low fit individuals (Wei et al. 1999, Lee et al.1999).

The physiological mechanisms whereby higher levels of fitness could confer such benefits are not fully understood and the interactions of fitness – fatness on nontraditional cardiovascular risk factors as inflammatory proteins have not been completely explored. Because low cardiorespiratory fitness and increased adiposity often occur in combination, masking their independent effects, it is unclear whether lower fitness or higher body fatness exerts a greater influence on inflammatory risk markers. A healthier immunological profile in subjects with higher physical activity and healthier fitness could simply be due to a generally leaner body composition in active subjects.

Obesity is associated with other risk factors, eg, hypertension, dyslipidemia, and diabetes, which could also increase the obesity associated inflammation, and adult populations that often present a clustering of potential confounding variables can hamper the drawing of conclusions about the relative contributions of body fatness and fitness to the CVD risk factor profile. Associations between obesity and inflammation in healthy young populations without other risk factors could be more reliable unbiased information.

Dividing the population in non-overweight and overweight, and fit and unfit, we observed in Study IV that the percentage of individuals with clinically relevant elevations generally decreases in subjects with higher fitness, and increases with overweight. Figure 2 shows mean values across fitness-fatness levels. Odds for having a clinically relevant elevation (upper age and gender-adjusted quartile) of CRP, ceruloplasmin, C3 or C4 was calculated, and for all variables, the greatest risk of having a clinically relevant value was found in the overweight, low-fitness category, except for ceruloplasmin and C3 in females, which showed the highest values in the overweight, high-fitness group.

Figure 2. Values of CRP, ceruloplasmin, C3 and C4 across levels of fitness and weight status. Fat-fit category NH corresponds to normal weight-high fitness, NL to normal weight-low fitness, OH to overweight-high fitness and OL to overweight-low fitness. P for trend was derived from ANOVA analysis of log transformed values of CRP, and square rooted values of C3, C4 and ceruloplasmin.

However, exploring the BMI-independent association between fitness and inflammation in regression models, we did not find any direct, independent association between inflammatory markers and fitness in any of the genders, but for C3 in males, suggesting that modulation of inflammatory status is mediated rather by body fat than by fitness status. These negative results could, however, be explained by a relatively high level of inactivity in this population. Nevertheless, we found a small but BMI independent relation between VO_2max and C3, but only in boys. This gender difference between fitness and inflammation interactions, has been observed before among children and young adults, where an increased cardiorespiratory fitness was associated with decreased CRP levels in boys (r=-0.32, p<0.01) but not in girls (r=-0.15, p=NS) (Isasi et al. 2003). A gender interaction between CRP and physical activity or fitness has also been observed in adults (Albert et al. 2004). We can speculate that this difference could be explained by more muscle mass in males, or that females are more inactive.

An interesting research area that could increase our understanding of inflammatory processes in relation to physical activity is the recognition of skeletal muscle as yet another endocrine organ. Muscle contractions without trauma, such as seen during moderate physical activity, stimulates the production and release of cytokines to the circulation (Febbraio & Pedersen, 2002). This modulation of cytokine balance could suppress pro-inflammatory activity at distant sites as well as within skeletal muscle, and considering that the skeletal muscle is the largest organ in the body, the molecular explanation by which we can understand how exercise mediates some of the health beneficial effects in relation to chronic disorders associated with systemic low-level inflammation, could be of great importance.

Deeper studies are required to clarify the indirect associations between fitness and inflammation in young, healthy populations. We will therefore focus, in future studies, on the relationships between data on both cardiovascular fitness and objectively measured physical activity (for total amount of muscle contractions) with acute phase proteins, but also with a panel of pro and anti-inflammatory cytokines in serum, reflecting all sources of cytokines, not only white blood cells.

4.4   Study strength and limitations

For the first time, a large national cross-sectional study has been performed in Spain to evaluate nutritional status and physical activity in adolescents. The large number of subjects included gives strength to the obtained data, and reference data are being published for future comparisons within this minority population. The use of immunological parameters to assess nutritional status in epidemiological studies is not common, but yet is widely recognized as a powerful tool to detect subclinical nutritional diseases, both by deficits or by excess. Its limited use in nutritional assessments is due to the need for immunology experts, a specialized laboratory and relatively high costs. The multidisciplinary view of the nutritional evaluation within the AVENA study gives this study an important strength as many confounders can be accounted for, and a broader view of adolescent health is produced, with nutritional status in focus.

Our study has some limitations. Because of our cross sectional design, we cannot establish the directionality of the associations. While the general assumption is that inflammation is consequent to increased adipose compartment, adipocyte hypoxia, and the stimulation of angiogenesis (Trayhurn & Woods 2004), it has been suggested that obesity is in fact a result of inflammatory disease (Das 2001).

Further, we used a single blood measurement of inflammation that may not accurately reflect long-term inflammatory status. Although we asked the subjects about any clinical infection during the study and no subject with a known underlying cause of infection was included, we can not rule out that elevated concentrations may have been due to the onset of an infection. The effect of any such measurement bias should be attenuated by the large numbers of participants enrolled. However, to further minimize the confounder of an ongoing infection, 9 subjects with CRP >10mg/L were excluded from this analysis. It's also important to stress that the levels of inflammatory markers shown between groups in this study are always within normal ranges, which means we must regard the whole sample as healthy, and the moderately but significantly higher inflammatory markers in overweight and obese adolescents may imply a chronic state of inflammation more clinically relevant in the long term.

5 CONCLUSIONS

- Reference data and percentile values of body fat composition in Spanish adolescents are presented. These data will contribute to future classification of adolescents in comparison with this well-established reference population, and to estimate the proportion of adolescents with high or low amounts of adiposity.

- Overweight and obesity during adolescence is associated with moderately raised serum concentrations of the major acute phase inflammatory protein C-reactive protein, which indicates the presence of a state of chronic low-grade inflammation in this population.

- Anthropometric measures of body fat are associated with acute phase proteins (C-reactive protein, ceruloplasmin and complement factors C3 and C4), suggesting a proinflammatory action of the adipose tissue in apparently healthy adolescents. Moreover, central obesity is an independent determinant of C3 levels.

- Overweight seems to be associated with low-grade systemic inflammation independently of fitness status, suggesting that modulation of inflammation is mainly mediated by body fat rather than fitness status. Complement factor C3 however, was independently associated with fitness, which suggests that C3 could be a new biomarker or involved in the mechanisms of the health benefits of fitness.

6   ACKNOWLEDGEMENTS

Michael Sjöström, tutor. Thank you for all the opportunities you are giving me, and for your trust in me. I believe this is not the end but the very beginning of a long-lasting fruitful scientific collaboration.

Ascensión Marcos, co-tutor and coordinator of the AVENA study. You have initiated my passion for immunonutrition and your trust in me has opened the most wonderful world of exciting challenges. I am enormously thankful.

Luis Moreno, co-tutor. Thank you for your valuable support during the AVENA study and the writing of this thesis. I am proud of being a part of your future projects! You are doing a wonderful job.

Esther Nova, co-tutor and friend. Thank you for sharing your most critical view of my work!

The AVENA study group, for the great work we have done together, although the endless field work was exhausting! A special thanks to the AVENA-Granada group for your support, friendship and for sharing your knowledge in exercise physiology.

Laura Barrios and Olle Carlsson, for valuable statistical support. I promise I will study hard so that we in the future only will meet to discuss the most interesting questions!

Colleagues and friends at CSIC and Prevnut, for your helping hand and nice coffee breaks.

Family and friends. Thank you for your understanding when I am absent and deep diving in science. You mean a lot to me, always. I love you.

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