Tesis Doctoral

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Capacidad aeróbica, composición corporal y variables genéticas de apolipoproteínas en relación a riesgo lipídico-metabólico en adolescentes

[Aerobic fitness, body composition and their relation with common apolipoprotein gene variants on lipid-related metabolic traits in adolescents]

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Directores de la Tesis Doctoral "Capacidad aeróbica, composición corporal y variables genéticas de apolipoproteínas en relación a riesgo lipídico-metabólico en adolescentes" [Aerobic fitness, body composition and their relation with common apolipoprotein gene variants on lipid-related metabolic traits in adolescents]
De la que es autor José Luis Mesa Mesa

AUTORIZAN la presentación de la referida Tesis Doctoral para su defensa y mantenimiento de acuerdo con lo previsto en el Real Decreto 56/2005, de 21 de Enero, emitiendo el siguiente informe:

La presente Tesis Doctoral se compone de un conjunto de artículos científicos originales, realizados por el doctorando bajo nuestra supervisión. Dichos trabajos han sido pubicados o sometidos en revistas científicas con factor de impacto. Ello corrobora la calidad de la presente Tesis Doctoral Europea.

Y para que conste y surta sus efectos en el expediente correspondiente, expido la presente en

Granada, 1 de Junio de 2006

Fdo. Manuel J. Castillo Garzón

Ángel Gutiérrez Sáinz

Dedicado a mis padres, hermanos, y demás familiares. Ellos son la inspiración y el motor para mi constante esfuerzo y superación personal

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2858, es el número final de adolescentes incluidos en la presente Tesis Doctoral. Ellos son los auténticos artífices e inspiradores de la presente Tesis Doctoral.

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lista de publicaciones

Las siguientes publicaciones han sido derivadas, directa o indirectamente, de la presente Tesis Doctoral:

Estudios realizados en el Departamento de Fisiología, Universidad de Granada, Spain

MESA JL, RUIZ JR, ORTEGA FB, WARNBERG J, GONZÁLEZ-LAMUÑO D, MORENO LA, GUTIÉRREZ A, CASTILLO MJ. Aerobic physical fitness in relation to blood lipids and fasting glycaemia in adolescents. Influence of weight status. *Nutr Metab Cardiovasc Dis* 2006; 16(4): 285-293.

MESA JL, ORTEGA FB, RUIZ JR, CASTILLO MJ, HURTIG-WENNLÖF A, SJÖSTROM M, GUTIÉRREZ A. The importance of cardiorespiratory fitness for healthy metabolic traits in children and adolescents: the AVENA Study. *J Public Health* 2006; 14(3): 178-180.

MESA JL, ORTEGA FB, CASTILLO MJ, RUIZ JR, TRESACO B, CARREÑO F, BUENO M, GUTIÉRREZ A, MORENO LA. Anthropometric determinants of a clustering of lipid-related atherogenesis risk factors in overweight and non-overweight adolescents. Influence of cardiorespiratory fitness. *Ann Nutr Metab* (*accepted*).

MESA JL, GONZÁLEZ-LAMUÑO D, RUIZ JR, ORTEGA FB, CARREÑO F, GUTIÉRREZ A, CASTILLO MJ, GARCÍA-FUENTES M. [Aerobic fitness standards related to low metabolic risk in adolescents according to apolipoprotein gene variants]. *Rev Esp Cardiol* (*submitted*).

MESA JL. Exercise in later life to prevent cognitive impairment [e-letter]. *Brit Med J*, 25 January 2005. http://bmj.bmjjournals.com/cgi/eletters/330/7484/189#94086

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Estudios realizados en el Medical Research Council, Cambridge, UK

FRANKS PW, **MESA JL**, HARDING AH, WAREHAM NJ. Gene-lifestyle interaction on risk of type 2 diabetes. *Nutr Metab Cardiovasc Dis* (2006 *in press*).

MESA JL, ONG K, LUAN J, LOOS RJ, O'RAHILLY S, BARROSO I, WAREHAM NJ. Absence of evidence for the association between *LMNA* variants and type 2 diabetes or metabolic syndrome. *Manuscript.*

Estudios realizados en el Royal Melbourne Institute of Technology, Melbourne, Australia

FEBBRAIO MA, **MESA JL**, CHUNG J, STEENSBERG A, KELLER C, NIELSEN HB, KRUSTRUP P, OTT P, SECHER NH, PEDERSEN BK. Glucose ingestion attenuates the exercise-induced increase in circulating HSP72 and HSP60 in humans. *Cell Stress & Chaperones* 2004; 9: 390-396.

WATT MJ, HOLMES AG, STEINBERG GR, **MESA JL**, KEMP BE, FEBBRAIO MA. Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity in human skeletal muscle. *Am J Physiol* 2004; 287: E120-E127.

MESA JL, HOLMES AG, CAREY AL, CHUNG J, WATT MJ, FEBBRAIO MA. Prolonged interleukin-6 treatment improves glucose tolerance *in vivo* and does not result in activation of the NFκβ signalling pathway. (*Pending of publication*).

índice / index

Cuadro resumen de Tesis Doctoral

Qué es conocido	Qué aporta esta Tesis Doctoral
Existen estudios que muestran relaciones entre capacidad aeróbica y variables lipídicas en adolescentes. Sin embargo, no tenemos constancia de estudios que establezcan los niveles mínimos de capacidad aeróbica asociados a un perfil lipídico saludable.	En la presente Tesis Doctoral se establecen los niveles mínimos de capacidad aeróbica asociados a un perfil lipídico saludable en adolescentes varones.
IMC, perímetro de cintura y la ratio cintura/cadera han sido propuestos como sencillas medidas antropométricas relacionadas con factores de riesgo metabólico en niños y adolescentes. Sin embargo, algunos estudios han puesto de manifiesto la falta de precisión de estas medidas para estimar variables lipídico-metabólicas en población adolescente.	En la presente Tesis Doctoral se muestra la utilidad del pliegue suprailíaco en niños y de la ratio cintura/cadera en niñas como sencillas medidas antropométricas relacionadas con variables lipídico-metabólicas en adolescentes.
Determinados polimorfismos en los genes de apolipoproteínas *APOC3* y *APOE* influencian el perfil lipídico. Sin embargo, no tenemos constancia de estudios en adolescentes que analicen posibles interacciones entre dichos polimorfismos genéticos y condición física aeróbica en relación al perfil lipídico.	En la presente Tesis Doctoral analizamos en adolescentes posibles interacciones entre condición física aeróbica y polimorfismos en los genes APOC3 y APOE en relación al perfil lipídico.
Varios estudios en adolescentes han analizado las relaciones existentes entre capacidad aeróbica, composición corporal y parámetros indicativos de estado inflamatorio. Sin embargo, no tenemos constancia de la existencia de estudios que analicen interacciones entre dichas variables y polimorfismos genéticos de apolipo-proteínas.	En la presente Tesis Doctoral mostramos evidencia de que la producción de citoquinas en células mononucleares está modulada por el polimorfismo SstI de APOC3 en adolescentes. También mostramos que dicha producción de citoquinas es inversamente proporcional al riesgo lipídico-metabólico, independientemente de la capacidad aeróbica y la masa corporal.
El ejercicio físico pude tener una influencia favorable sobre factores de riesgo cardiovascular tales como diabetes, hypertension, obesidad, dislipidemias, o disfunción endotelial. Los mecanismos moleculares de tales efectos no están completamente claros, aunque sí se sabe que dichas mejoras pueden ser debidas a incrementos en la lipolisis, translocación de GLUT4, o capacidad antioxidativa.	ANEXO En la presente Tesis Doctoral planteamos la posibilidad de que las mejoras metabólicas (principalmente en la homeostasis de la glucosa) inducidas por ejercicio físico puedan ser mediadas, al menos en parte, por los incrementos plasmáticos de proteínas Heat Shock y de interleuquina-6.

PhD Thesis summary box

What is already known on this topic	What this PhD Thesis adds
There are studies showing associations between physical fitness and lipid variables in adolescents. However, to our knowledge there are no studies providing minimal criterion standards of aerobic fitness in adolescents associated to healthy lipid-related metabolic variables.	We set minimal levels of aerobic physical fitness associated to a favourable lipid profile in male adolescents.
BMI, waist circumference, and waist-to-height ratio have been proposed as simple anthropometric measures related to metabolic and cardiovascular risk factors in children and adolescents. However, there are some studies casting some doubts about the value of these measurements to accurately explain the variability of lipid-related metabolic variables.	We demonstrate the usefulness of suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk in adolescents.
Common apolipoprotein variants of *APOC3* and *APOE* genes influence lipid-related metabolic traits. However, to our knowledge there are no studies analysing possible interactions between these genes and physical fitness in relation to lipid-related metabolic traits in adolescents.	We provide genotype-dependent aerobic physical fitness levels associated to a favourable lipid-related metabolic profile in adolescents.
Several studies in adolescents have assessed complex relationships between fitness, fatness and inflammation. However, to our knowledge there are no studies in this population analysing possible interactions with common apolipoprotein gene variants.	We suggest that cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents, regardless physical fitness or weight status.
Physical exercise can positively influence classical cardiovascular risk factors such as diabetes, hypertension, obesity, dyslipidemias, and endothelial dysfunction.	ANNEX
The physiological and molecular mechanisms are not fully understood, although increased lipolysis, GLUT4 translocation, and antioxidant capacity have been reported with physical exercise.	We provide the possibility that exercise may induce beneficial metabolic effects (mainly in glucose homeostasis) through acutely and systemically increased heat shock proteins and interleukin-6.

resumen general

Introducción

La enfermedad cardiovascular es la primera causa de muerte en países desarrollados. Junto con la enfermedad cardiovascular, la obesidad es uno de los problemas de salud pública más acuciantes, incluso en países menos desarrollados. Los problemas de obesidad, sobrepeso y enfermedades metabólicas derivados de la obesidad están alcanzando una dimensión global durante los últimos 10 años. Durante este tiempo, la incidencia de complicaciones metabólicas derivadas de la obesidad se ha incrementado considerablemente. Cabe destacar que dicho incremento ha sido mayor en niños y adolescentes que en población adulta. Puesto que los procesos etiológicos ligados a obesidad desencadenantes de complicaciones metabólicas comienzan durante la adolescencia, en la presente Tesis Doctoral se han realizado una serie de estudios en población adolescente, con los siguientes objetivos:

1) Proveer medidas sencillas de condición física y composición corporal capaces de estimar un índice global de riesgo lipídico-metabólico en adolescentes.
2) Analizar interacciones entre variantes genéticas de apoproteínas y condición física en relación a variables lipídico-metabólicas.

Métodos

Para analizar posibles asociaciones entre condición física aeróbica (determinada mediante el test de Course Navette), medidas antropométricas y variables de riesgo metabólico, usamos la población del estudio AVENA (Análisis y Valoración del Estado Nutricional en Adolescentes). Esta población consistió en 3000 adolescentes (13-18 años) de cinco diferentes áreas geográficas de España. Los sujetos completaron los tests de condición física de la batería Eurofit, así como una evaluación antropométrica completa. Además, en 600 sujetos seleccionados aleatoriamente de la muestra principal, se analizó de forma completa el perfil lipídico.

Para analizar posibles interacciones entre variantes genéticas de apoproteínas y condición física en relación a riesgo metabólico, usamos la población del estudio AVENA. Las variables genéticas de apoproteínas fueron seleccionadas en base a estudios publicados previamente.

Resultados generales

Condición física y medidas antropométricas en relación a riesgo lipídico-metabólico

Tanto la condición física aeróbica (medida mediante el test de Course Navette) como el índice de masa corporal se relacionaron con un índice global de perfil lipídico y glucemia en adolescentes. En adolescentes masculinos se calcularon los niveles mínimos de condición física aeróbica asociados a un perfil lipídico saludable. Sólo el 50% de la población masculina adolescente alcanzó dichos valores de condición física aeróbica. Con respecto a medidas antropométricas, el pliegue suprailiaco en niños y la ratio abdomen/cadera en niñas fueron los índices que más se relacionaron con un índice general de perfil lipídico, por encima del índice de masa corporal. Estas asociaciones se daban fundamentalmente en niños sin sobrepeso, y fueron independientes del estado de condición física de los adolescentes.

Interacciones entre variables genéticas de apoproteínas y condición física aeróbica en relación a variables lipídico-metabólicas

En función de variantes genéticas de apolipoproteínas fueron obtenidas asociaciones entre la capacidad física aeróbica y variables lipídico-metabólicas. Para presentar el mismo índice de riesgo lipídico-metabólico, los portadores del alelo S2 del gen *APOC3* o del alelo ε4 de *APOE* (33% de la población analizada) debían tener mayor capacidad aeróbica que los portadores de los genes *APOC3* S1/S1 o *APOE* ε3/ε3. Los portadores del alelo S2 de *APOC3* presentaron también una disminución en la liberación de IFN-γ, IL-10, IL-6, IL-2 y TNF-α de células mononucleares, que a su vez se vio asociado a un incremento del riesgo de presentar un elevado riesgo lipídico-metabólico.

Conclusiones

Nuestros datos confirmam la importancia de la condición física aeróbica y el control de peso en relación a variables lipídico-metabólicas en adolescentes. Ello sugiere que un buen control sobre la condición física y el peso corporal podría ayudar a prevenir el riesgo metabólico en adolescentes, y que dicho control se vería optimizado teniendo en cuenta variables genéticas de apoproteínas.

Sería de interés que los valores de referencia mínimos de condición física aeróbica establecidos en la presente Tesis Doctoral se adoptaran en colegios e instituos españoles como valores deseables en la población adolescente. Esto ayudaría a mantener unos mejores niveles de salud metabólica, tanto en la propia población adolescente como cuando ésta se convierta en población adulta.

general summary

Introduction

Cardiovascular disease is the leading cause of death in developed countries. In addition, obesity is one of the most blatantly visible, yet most neglected, public-health problems that threaten to overwhelm both more and less developed countries. The problems of overweight, obesity, and obesity-related metabolic complications have achieved global recognition only during the past 10 years. During this time the incidence of obesity- related metabolic complications has increased considerably. Of note, the increases in the prevalence of both overweight and obesity have been greater in children and adolescents than in adults over the same time frame. Since the pathological processes linked to obesity and leading to further metabolic risk occur during adolescence, in this PhD Thesis a series of studies, mainly focused on this population, were conducted:

1) Studies aimed at providing simple measures of physical fitness and body composition associated to metabolic traits in adolescents.
2) Studies aimed at testing whether physical fitness levels were associated with lipid metabolic traits in an apolipoprotein genotype-dependent fashion.

Methods

To test for associations between physical fitness, anthropometric measures and metabolic traits, we used the cohort of the AVENA (Análisis y Valoración del Estado Nutricional en Adolescentes [Assessment of Nutritional State in Spanish Adolescents]) study. This population consisted on 3000 adolescents (13-18 yr) from five different geographic areas of Spain. Individuals completed a complete physical fitness evaluation (using the Eurofit battery), as well as a complete anthropometric assessment. In addition, a comprehensive lipid profile was analysed in 600 randomly chosen individuals.

To test whether physical fitness levels were associated with metabolic traits in an apolipoprotein genotype-dependent fashion, we used the cross-sectional population of the AVENA study in Spain. Common genetic variants previously reported to be associated with metabolic traits were genotyped in our adolescent population from the AVENA study, and possible interactions with physical fitness and anthropometric measures in relation to lipid-related metabolic traits were tested.

Main findings

Physical fitness and anthropometric measures in relation to lipid-related metabolic traits

Both aerobic physical fitness (by using the widely used Course Navette [Shuttle Run] test) and weight status were associated to metabolic traits (a composite index of blood lipids and glycaemia) in adolescents. Standard aerobic fitness levels associated to healthy lipid profile were calculated in males. Only 50% of the male population reached the required physical fitness values associated to a healthy lipid profile. Regarding anthropometric measures, we found suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk, mainly in non-overweight adolescents and regardless their physical fitness status.

Apolipoprotein gene variants and physical fitness in relation to lipid-related metabolic traits

We found genotype-dependent associations between physical fitness and metabolic traits in adolescents. According to common apolipoprotein genetic variants (*APOC3* SstI polymorphism and *APOE* isoforms) we determined the required physical fitness levels associated to a healthy metabolic profile in adolescents. In addition, carriers of the S2 allele of *APOC3* presented a lower cytokine profile production from peripheral blood mononuclear cells. This association remained after adjustment for confounding factors (including physical fitness and weight status). Interestingly, this low cytokine production (mainly tumour necrosis factor-α and IL-6) was associated to a higher lipid-related metabolic risk.

Conclusions

Our data suggest that both aerobic fitness and weight management may be necessary for the prevention of metabolic risk in adolescents.

We also provide, according to common apolipoprotein genetic variants, the minimal criterion standards of aerobic fitness to present a healthy plasma lipid profile in male adolescents. This is a new tool which should be adopted by schools as "aerobic fitness standards" in order to keep a healthy plasma lipid profile and, thus, to prevent metabolic and cardiovascular risk during adolescence and later in life.

Future studies are warranted to explore the connections between genes, exercise, physical fitness, body composition and metabolic status to improve the health of both adolescent and adult populations in the XXI century.

1. general introduction

1.1. Paediatric and adolescent obesity: its coincidence with low physical activity

Paediatric and adolescent obesity represents an uncontrolled and increasing worldwide epidemic (Ogden et al., 2002; Ebbeling et al., 2002; Caroli, 2003; Hedley et al., 2004; Moreno et al., 2005). Further, the increases in the prevalence of both overweight and obesity have been greater in children and adolescents than in adults over the same time frame. Even in developing countries, the rates of overweight and obesity are increasing rapidly (Guillaume & Lissau, 2002). However, the prevalence of paediatric obesity differs a lot between different studies (Lissau, 1997) and between different countries (Lissau et al., 2004). Differences in methodology and year of survey may explain some of the differences. In a recent paper, this problem was overcome by performing the data collection using an identical survey design in all the participating countries within an already-running comparative study (Health Behaviour in School Children (HBSC)) (WHO, 2000). The prevalence of obesity in US children and adolescents has increased dramatically in the last decades (Ogden et al., 2002). In Europe, there are little representative data about obesity prevalence in adolescents and the existing ones are not comparable, because different definitions for obesity were used. However, available results point out that there is also a dramatic increase in the prevalence of obesity in European adolescents (Moreno et al., 2001). Overweight and obesity prevalences in the adolescent Spanish population are increasing (Moreno et al., 2000; 2002). At European level, Spain has one of the highest adolescent obesity prevalences, and is experiencing alarmingly increasing rates of changes.

Concern about increased levels of childhood and adolescent overweight and obesity and whether this pattern will continue into adulthood underscores the importance of being physically active (Sallis et al., 2000). However, studies report a decline in physical activity levels during adolescence (Frankish et al., 1998; Allison et al., 1999). In addition, Deforche et al. (2006) demonstrate that overweight and obese adolescents show lower sport participation and have a less positive attitude toward physical activity. These studies, taken

together, underscore the importance of physical activity in the prevention of overweight and obesity in childhood and adolescence.

This epidemic rise in obesity is a great challenge for public health and for decision makers in the health system. It highlights the importance of prevention studies and makes it clear that it is very important to initiate prevention before adulthood, i.e. during childhood or adolescence. In order to know how to prevent childhood obesity, it is important to know the risk factors involved.

1.2. Overweight-derived metabolic complications in adolescence

Long-term health complications in overweight children and adolescents include atherosclerosis and increased rates of cardiovascular diseases and mortality (Mossberg, 1989; Must et al., 1992). In North-America, about 10% of 12- to 19-year olds have total cholesterol levels exceeding 200 mg/dl (Hickman et al., 1998). Rates of cardiovascular diseases and diabetes have been found to increase in both men and women who were obese during adolescence (Dietz, 1998). Approximately 50% of obese adolescents with a body mass index at or above the 95th percentile become obese adults (Dietz, 1998).

Atherosclerosis starts in childhood and adolescence (Berenson et al., 1998; Strong et al., 1999; Knoflach et al., 2003), but clinical manifestations can appear 30 to 50 years later. This is the reason why it is so important to identify risk factors as early as possible. Risk factors for several of the major chronic diseases, such as cardiovascular diseases, hypertension, diabetes, obesity, and cancer, are often observed during childhood.

These studies underscore the importance of preventing overweight-derived metabolic complications from childhood and adolescence.

1.3 Necessity of early identification and prevention of overweight-related disorders in adolescence

The continuing upward trends in the prevalence of child obesity and its related metabolic disorders indicate the need for early identification and characterization of the relationship between simple measures of obesity and both cardiovascular and metabolic complications in adolescents (Rodríguez et al., 2004; Steinbeck & Pietrobelli, 2005). Aerobic physical fitness, anthropometric characteristics, and inflammatory markers are among these measures.

Aerobic physical fitness

Increased aerobic physical fitness (which is in part the result of regular practice of physical activity) during adolescence has been associated not only with healthier lipid-related metabolic traits during these years (Bergström et al., 1997; Boreham et al., 2001), but also later in life (Twisk et al., 2002). In an 8-year follow up study (Hasselstrøm et al., 2002), aerobic physical fitness during adolescence was not associated to risk factors of cardiovascular disease in adulthood, but changes from the adolescence to adulthood in aerobic fitness were related to risk in adulthood. Moreover, subjects who decreased their aerobic fitness levels also changed to a worse risk factor profile (Hasselstrøm et al. 2002). Changes in aerobic fitness from adolescence to adulthood were also inversely and significantly associated with large arterial stiffness (a major risk factor for cardiovascular disease) (Ferreira et al. 2003; Boreham et al 2004).

These studies underscore the importance of aerobic physical fitness to prevent cardiovascular risk in adolescents and later in life. However, at the present time and to our knowledge, there are no studies providing minimal criterion standards of aerobic fitness associated to healthy lipid-cardiovascular profile in adolescents. Given the importance of common apolipoprotein genetic variants of *APOC3* and *APOE* genes on lipid-related metabolic traits (Bernstein et al., 2002; Perez-Jimenez et al., 2002), it would be interesting to set such criterion standards according to these genetic variants.

Anthropometric and inflammatory markers

Long-term health complications in overweight children and adolescents include atherosclerosis and increased rates of cardiovascular diseases and mortality (Must et al., 1992). In fact, subtle plasma lipid abnormalities predisposing to atherosclerosis are already detected in overweight children and adolescents (Kang et al., 2002; Weiss et al., 2004). Body mass index (BMI) has been proposed as a simple anthropometric measure related to cardiovascular risk factors in children and adolescents (Katzmarzyk et al., 2004). However, other studies highlight important shortcomings of the BMI measurement to provide information on body fat distribution, thereby masking true obesity-related metabolic and cardiovascular risk in children (Savva et al., 2001). Therefore, it would be interesting to study other anthropometric measures in relation to lipid-cardiovascular risk in adolescents.

Evidence suggests that inflammatory processes and the immune response may play an important role in the early development of both cardiovascular disease and diabetes (Greaves & Channon, 2002; Kolb & Mandrup-Poulsen, 2005). Given the uncontrolled and increasing worldwide obesity epidemic and related metabolic disorders in children and adolescents (Ebbeling et al., 2002; Hedley et al., 2004; Moreno et al., 2005), studies analysing a possible role of the immune system on obesity-related metabolic disorders in adolescents are warranted.

1.4 General aims

The general aims of this PhD Thesis are:

1) To provide simple physical fitness and anthropometric measures associated with obesity-related metabolic traits in adolescents. These measures might be used as early identification to prevent obesity and metabolic risk in the crucial stage of adolescence.

2) To analyse possible apolipoprotein gene variants and physical fitness interactions in relation to lipid-related metabolic traits. This might be useful to promote more individualized lifestyle programs to prevent metabolic risk.

3) To examine possible interactions between physical fitness, weight status, immune system and apolipoprotein gene variants on lipid-related metabolic traits. This might be useful to clarify the role of immune response in overweight-derived metabolic traits.

4) In the annex of this PhD Thesis we intend to provide possible and novel physiological mechanisms able to explain, at least in part, the exercise-induced beneficial metabolic effects.

2. general methodology

In this PhD Thesis we used the sample of the AVENA (Alimentación y Valoración del Estado Nutricional en Adolescentes: Food and assessment of the Nutritional Status of Adolescents). This was a Spanish multicenter study designed to evaluate the nutritional status of a geographically representative sample of Spanish adolescents in order to identify risk factors for chronic and metabolic 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) #00/0015.

The general methodology of the study, as well as the sample inclusion criteria, has been published elsewhere (González-Gross et al., 2003; Moreno et al., 2003).

2.1. Study population

The AVENA study was performed on 2851 Spanish adolescents (1354 males and 1497 females) aged 13 to 18.5 years were selected by means of a multiple-step, simple random sampling, taking into account the location (Granada, Madrid, Murcia, Santander and Zaragoza) and random assignment of the schools within each city.

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

The sample size was stratified by age and sex. In order to guarantee a representative sample of the whole country, we calculated the number of adolescents to be included in the study by selecting the variable with the greatest variance for this age group 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 ±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. 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 fulfil the inclusion criteria were excluded. Exclusion criteria were: type 2 diabetes, pregnancy, alcohol or drug abuse, and nondirectly-related nutritional medical conditions.

Socioeconomic status was assessed by examining paternal educational level and occupation. The subjects were accordingly classified into five categories: low, medium-low, medium, medium-high and high socioeconomic status.

2.2. Ethics

Parents or supervisors were informed by a letter about the nature and purpose of the study and they gave their written informed consent. The study protocol was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki (Hong Kong revision, September 1989), following the ECC Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law which regulates clinical research in humans. The study protocol was approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

2.3. 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 Granada was responsible for biochemical, fitness and physical activity assessments. The University of Zaragoza was responsible for body composition assessment. The University of

Cantabria (Santander) was responsible for genotyping assessment, and the University of Murcia for dietary records.

All methods used in the AVENA study are summarized in Tables 1 and 2. In this PhD Thesis we included the assessment of lipid profile and related metabolic traits, genotype polymorphisms, body composition, sexual maturation, physical fitness, and cell-mediated immunity markers. Blood variables analyzed in the subgroup with blood samples are summarized in Table 2.

Table 1. Variables studied within the AVENA study. *Assessed in a subgroup of 238 subjects (AVENA-Zaragoza city only)

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 (VO2max 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)

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)

2.4. Anthropometric assessment

For anthropometric measurements, subjects were barefoot and in their underwear. Weight was measured with a Seca scale (precision of 50 g), and height with incorporated stadiometer to the scale (precision of 1 mm). Biceps, triceps, subscapular, suprailiac, thigh, and calf skinfold thicknesses were measured with a Holtain lipocaliper (range 0–40 mm, precision of 0.1 mm). Arm, thigh, waist and hip circumferences were measured with an inelastic tape (precision of 1 mm).

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 used to assess body composition within the AVENA multicenter study was strictly controlled and has been previously published elsewhere (Moreno et al., 2003, 2005). To establish the overweight (including obesity) and non-overweight categories, we used the gender- and age-adjusted cut-off points provided by the International Obesity Task Force (Cole et al., 2000). 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.

2.5. Aerobic physical fitness assessment

The physical fitness status of the AVENA population was evaluated using tests, most of them from EUROFIT, the European test of physical fitness. A detailed description of the EUROFIT tests is given in Oja and Tuxworth (1995). The battery of fitness tests used in the AVENA study included the assessment of the following fitness measures: whole body balance, speed of limb movement, flexibility, explosive strength, hand-grip strength, upper body muscular endurance, running speed & coordination, and aerobic physical fitness (cardiorespiratory fitness). In this PhD Thesis we focussed on cardiorespiratory fitness. Cardiorespiratory fitness was measured by the progressive 20-m shuttle run test (Léger & Lambert, 1982), which has been validated for use in children (Boreham et al., 1990).

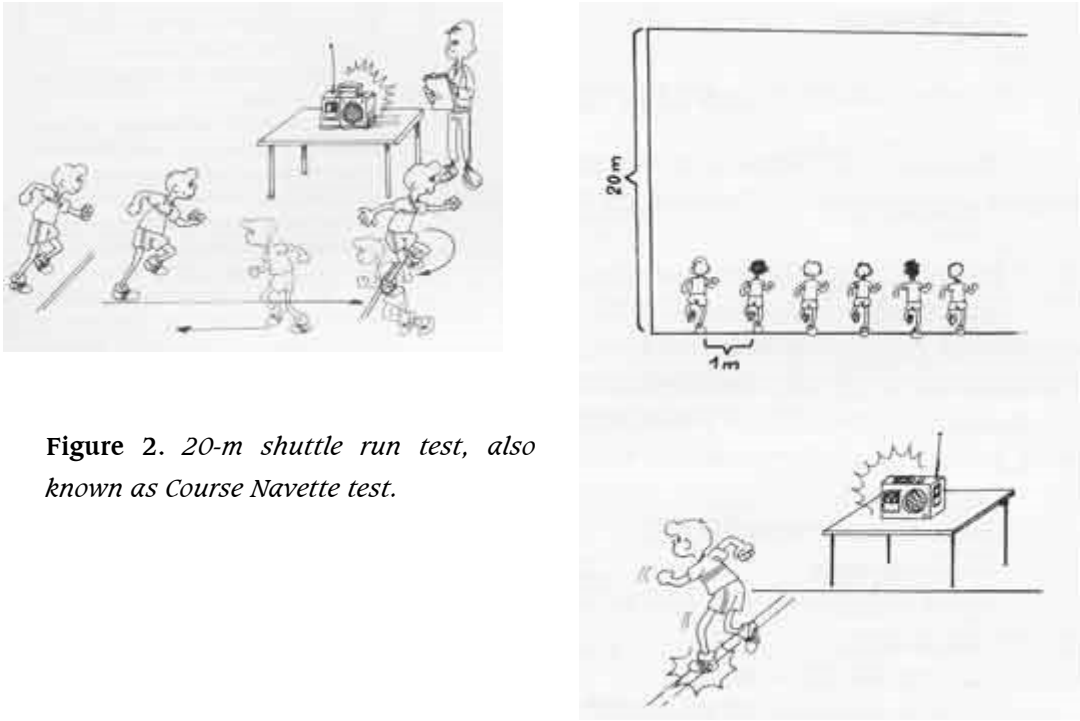

Figure 2. *20-m shuttle run test, also known as Course Navette test.*

This test required subjects to run back and forth between two lines set 20 m apart. Running pace was determined by audio signals, emitted from a pre-recorded cassette tape, the initial

velocity being 8.5 km·h-1, and increasing by 0.5 km·h-1 every minute (step). The tape used was calibrated over 1 min duration and the tape machine was checked for accuracy prior to each test. Subjects were instructed to run in a straight line, to pivot upon completing a shuttle and to pace themselves in accordance to the time intervals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on two consecutive occasions.

2.6. Laboratory methods

Blood sampling

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). Blood collection was carried out between 8:00 and 9:00 a.m., and after an 8-h overnight fast. The subjects were selected randomly (according to age, sex, geographical location, and economic status) and were instructed to abstain from alcoholic beverages for at least two weeks before sampling and to refrain from vigorous exercise during the 48 h preceding blood collection. Within one hour after collection, blood was centrifuged and aliquots of sera were sent refrigerated to a central laboratory (Clinical Biochemistry Service, Granada University Hospital) where all the clinical chemistry tests were performed within 24 hours after collection.

Lipid profile assessment

Plasma glucose, total cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol were measured by enzymatic assay using a Hitachi 911 Analyzer (Roche Diagnostics, Indianapolis, Ind, USA). For the HDL cholesterol assay, precipitation was done using reagents provided by Boehringer (Ingelheim, Germany). LDL cholesterol was calculated with the Friedewald formula (Friedewald et al., 1972) adjusted for plasma triglycerides levels (Nakanishi et al., 2000). Apolipoprotein (apo) A-I, apo B-100 and lipoprotein(a) [Lp(a)] were measured using a immunonephelometric assay on Array 306 system (Beckman GMI, Inc., Albertville, Minnesota, USA).

In vitro production of cytokines

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. 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.

Genotyping

Genomic DNA was isolated form leukocytes of whole blood samples according to standard procedures (Miller 1988). The extracted DNA was stored at -70°C until analysed. *APOE* genotyping was performed by means of polymerase chain reaction (PCR), specific digestion with H*hal* (Promega), and electrophoresis in 12% acrylamida gel. Genotype characterization for the polymorphic S*stI* (position 371) in the 3' untranslated region of the *APOC3* gene was performed by PCR, specific digestion of the amplified products with the restriction enzyme SstI (Promega), and electrophoresis in 2% agarose gel (Hixson et al., 1991).

2.7. Statistics

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

Inter-groups differences among gender were assessed by either Student's test (parametric variables) or Mann Whitney U test (non-parametric variables).

General linear modelling was used to test associations between aerobic fitness, blood lipids, and glycaemia, after adjustment for confounding factors. ROC analysis was used to summarize the potential diagnostic accuracy of aerobic fitness to discriminate between the presence/absence of a favourable lipid profile. Multicollinear analysis and generalized linear

modelling were used for assessing the relationship between body composition variables (BMI, waist-to-hip ratio, skinfold thicknesses and circumferences) and the lipid-related metabolic risk score. To test the interaction between anthropometric variables and cardiorespiratory fitness on the metabolic risk score, further linear modelling was undertaken.

Generalized linear model analyses adjusted for confounding factors were used to assess the associations between cytokine production from PBMCs and a lipid-related cardiovascular risk score. To test the interaction between anthropometric variables, cardiorespiratory fitness, and genetic polymorphisms on both cytokine production from PBMCs and the cardiovascular risk score, further linear modelling was undertaken.

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3.

Aerobic fitness and body composition in relation to lipid-related metabolic traits

What is already known on this topic

There are studies showing associations between physical fitness and lipid variables in adolescents. However, to our knowledge there are no studies providing minimal criterion standards of aerobic fitness in adolescents associated to healthy lipid-related metabolic variables.

BMI, waist circumference, and waist-to-height ratio have been proposed as simple anthropometric measures related to metabolic and cardiovascular risk factors in children and adolescents. However, there are some studies casting some doubts about the value of these measurements to accurately explain the variability of lipid-related metabolic variables.

What this PhD Thesis adds

We set minimal levels of aerobic physical fitness associated to a favourable lipid profile in male adolescents.

We demonstrate the usefulness of suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk in adolescents.

This section comprises two studies analysing aerobic physical fitness and anthropometric measurements in relation to lipid-related metabolic traits:

Aerobic physical fitness in relation to blood lipids and fasting glycaemia in adolescents. Influence of weight status.

MESA JL, RUIZ JR, ORTEGA FB, WARNBERG J, GONZÁLEZ-LAMUÑO D, MORENO LA, GUTIÉRREZ A, CASTILLO MJ. Published in *Nutr Metab Cardiovasc Dis* 2006; 16(4): 285-293.

Anthropometric determinants of a clustering of lipid-related metabolic risk factors in overweight and non-overweight adolescents. Influence of cardiorespiratory fitness.

MESA JL, ORTEGA FB, CASTILLO MJ, RUIZ JR, TRESACO B, CARREÑO F, BUENO M, GUTIÉRREZ A, MORENO LA. *Ann Nutr Metab* 2006 (*accepted*).

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3.1.

Aerobic physical fitness in relation to blood lipids and fasting glycaemia in adolescents: Influence of weight status

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KEYWORDS
Adolescence;
Aerobic fitness;
Blood lipids;
Weight status;
Atherosclerosis;
Cardiovascular disease;
Cardiovascular prevention

Abstract *Background and aims:* We explored the associations between aerobic physical fitness with blood lipids and a composite index of blood lipids and fasting glycaemia in adolescents, analysing possible interactions with weight status.
Methods and results: Body mass index and aerobic physical fitness was measured in 2090 adolescents (1034 males and 1056 females) 13–18.5 years by using the 20-m shuttle run test. Plasma glucose, total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol, triglycerides, apolipoprotein (apo) A-I, apo B-100 and lipoprotein(a) [Lp(a)] were measured in 460 of the 2090 subjects. After adjustment for confounding factors, a continuously distributed summary score for blood lipids and fasting glycaemia was significantly related to aerobic fitness in males ($P = 0.018$) and females ($P = 0.045$, from the 2nd to the 4th quartile of aerobic fitness). After adjustment for gender, age, sexual maturation and economic status, aerobic fitness was related to the composite index of blood lipids and glycaemia in both overweight and non-overweight adolescents ($P < 0.05$). However, for the same level of aerobic fitness, the composite index of blood lipids and glycaemia was significantly higher in overweight adolescents ($P = 0.001$). After setting the minimal

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aerobic fitness standards to present a healthy lipid profile, about 50% of males did not reach such values.
Conclusion: Our data suggest that both aerobic fitness and weight management are associated with a composite index of blood lipids and glycaemia in adolescents. Our study also provides the minimal levels of aerobic physical fitness associated with a favourable lipid profile in male adolescents, a new tool which should be adopted by schools as ''aerobic fitness standards''.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in developed countries. Although the clinical manifestations of CVD occur in middle adulthood, pathological data have shown that atherosclerosis begins in childhood and adolescence [1—3]. Disturbed plasma lipid profile is an important cardiovascular risk factor, capable of inducing atherosclerotic development [4,5]. This has been early shown in adults, but holds also for children and adolescents. In fact, it has been recently demonstrated that plasma low density lipoprotein (LDL) cholesterol levels measured in childhood are a consistent predictor of carotid artery intima-media thickness in young adults who are still too young to experience coronary events [6,7]. These, and other findings [8,9], suggest that a primary goal in CVD prevention should be to keep a healthy plasma lipid profile since childhood [10,11].

Fasting glycaemia also deserves some attention. In fact, fasting glucose has been proposed as a marker of loss of beta cell function and insulin response [12], and there are noticeable similarities in the cardiovascular risk factor profile in subjects with impaired fasting glycaemia and in subjects with impaired glucose tolerance [13].

Regular aerobic physical activity leads to a significant cardiovascular risk reduction, by improving the plasma lipid profile [14,15]. Along the same line, increased aerobic physical fitness (which is in part the result of regular practice of aerobic physical activity) during adolescence has been associated not only with healthier blood lipids during these years [16,17], but also later in life [18]. Therefore, it seems reasonable to initiate regular aerobic physical activity in childhood in order to prevent metabolic risk and CVD in adulthood.

The previous studies did not analyse possible associations with a metabolic composite index, but with single blood lipids. Therefore, our first aim was to explore associations between aerobic physical fitness not only with single blood lipids, but also with a composite index of blood lipids and fasting glycaemia in adolescents. Of note, only one study [19] analysed interactions between obesity measures and aerobic physical fitness in relation to a metabolic composite index, so we also tested a similar interaction in our population. Finally, to the best of our knowledge there are no studies providing minimal criterion standards of aerobic fitness in adolescents, associated with healthy metabolic outcomes. Therefore, we secondly aimed to set minimal criterion standards of aerobic fitness associated with a favourable lipid profile in adolescents.

Methods

Study population and design

This research was part of the AVENA study (Análisis y Valoración del Estado Nutricional en Adolescentes españoles [Assessment of Nutritional Status in Spanish Adolescents]), a population-based cross-sectional multicentric study of the aetiology and pathogenesis of obesity and related metabolic disorders during adolescence. The general methodology of the study, as well as the sample inclusion criteria, has been published elsewhere [20,21]. Briefly, 2851 Spanish adolescents (1354 males and 1497 females) aged 13—18.5 years were selected by means of a multiple-step, simple random sampling, taking into account the location (Madrid, Murcia, Granada, Santander and Zaragoza) and random assignment of the schools within each city. The inclusion criteria were: (1) not to be a consumer of alcohol, drugs or steroids, (2) not to have familial hypercholesterolaemia, (3) no history of CVD, (4) to be free of disease and medication at the time of the study, and (5) not to be pregnant. Socioeconomic status was assessed by examining paternal educational level and occupation. The subjects were accordingly classified into five categories: low, medium-low, medium, medium-high and high socioeconomic status.

Parents or supervisors were informed by letter about the nature and purpose of the study and they gave their written informed consent. The

study protocol was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki, and approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

Aerobic physical fitness assessment

Aerobic fitness was measured by the progressive 20-m shuttle run test [22]. This test was validated for use in children about 20 years ago [23,24]. Since then, it has been used in schools worldwide to assess aerobic physical fitness in children and adolescents. Two thousand ninety adolescents (1034 males and 1056 females) completed satisfactorily the test (characteristics of the study population are shown in Table 1). This test required subjects to run back and forth between two lines set 20 m apart. Running pace was determined by audio signals, emitted from a pre-recorded cassette tape, the initial velocity being 8.5 km/h, and increasing by 0.5 km/h every minute (step). The tape used was calibrated over 1 min duration and the tape machine was checked for accuracy prior to each test. Subjects were instructed to run in a straight line, to pivot upon completing a shuttle and to pace themselves in accordance with the time intervals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on two consecutive occasions. The final score was computed as the number of steps completed (precision of 0.5 steps). A constant level of encouragement was given to participants throughout the test. Subjects were instructed to abstain from strenuous exercise in the 48 h preceding the test.

Table 1 Physical characteristics and lipid profile of the study population

	Boys ($n = 1034$)	Girls ($n = 1056$)	P
Age (years)	15.4 (1.3)	15.4 (1.3)	NS
Weight (kg)	63.8 (12.9)	56.3 (9.6)	<0.001
Height (cm)	171.2 (8.5)	161.9 (6.2)	<0.001
BMI (kg/m^2)	21.7 (3.5)	21.5 (3.3)	NS
Total cholesterol (mg/dl)	155.8 (26.0)	168.5 (26.4)	<0.001
Glucose (mg/dl)	96.4 (10.9)	92.0 (8.7)	<0.001
LDL cholesterol (mg/dl)	89.9 (23.4)	96.3 (23.8)	<0.01
HDL cholesterol (mg/dl)	51.5 (9.5)	59.2 (11.5)	<0.001
Triglycerides (mg/dl)	72.4 (31.9)	64.7 (26.9)	<0.01
Apo A-I (mg/dl)	116.8 (20.0)	126.4 (25.3)	<0.001
Apo B-100 (mg/dl)	67.4 (14.8)	70.8 (14.7)	<0.05
Lp(a) (mg/dl)	30.5 (36.3)	31.1 (39.6)	NS

Results expressed as mean (SD). Lipid and glucose data are from 460 subjects (248 males and 212 females). P values are from either Student's t-test (parametric variables) or Mann–Whitney U-test (non-parametric variables). Apo, apolipoprotein; BMI, body mass index; Lp(a), lipoprotein(a).

Anthropometric assessment

For anthropometric measurements, subjects were barefoot and in their underwear. Weight was measured with a Seca scale (precision of 50 g), and height with incorporated stadiometer to the scale (precision of 1 mm). Trained interviewers asked the adolescents to classify themselves in one of the five Tanner stages of pubertal maturity. This standard staging describes breast and pubic hair development in girls and genital and pubic hair development in boys. The first Tanner stage corresponds to the prepubertal state; subjects classified in Tanner stage 5 are completely mature. Harmonization and standardization of anthropometric measurements used to assess body composition within the AVENA multicentre study were strictly controlled and have been previously published elsewhere [21,25]. To establish the overweight (including obesity) and non-overweight categories, we used the gender- and age-adjusted cut-off points provided by the International Obesity Task Force [26].

Laboratory methods

Blood sampling

Blood was collected in 581 subjects 5 days before the 20-m shuttle run test. Four hundred and sixty (248 males and 212 females) out of the initial 581 subjects completed satisfactorily the aerobic fitness test. Blood collection was carried out between 8:00 and 9:00 a.m., and after an 8-h overnight fast. The subjects were selected randomly (according to age, gender, geographical location, and economic status) and were instructed to abstain from alcoholic beverages for at least 2 weeks before sampling, and to refrain from vigorous exercise during the 48 h preceding blood collection. Within 1 hour after collection, blood was centrifuged and aliquots of sera were sent refrigerated to a central laboratory (Clinical Biochemistry Service, Granada University Hospital), where all the clinical chemistry tests were performed within 24 h after collection.

Lipids, lipoproteins, and Lp(a)

Plasma glucose, total cholesterol, triglycerides and high density lipoprotein (HDL) cholesterol were

measured by enzymatic assay using a Hitachi 911 Analyzer (Roche Diagnostics, Indianapolis, USA). For the HDL cholesterol assay, precipitation was done using reagents provided by Boehringer (Ingelheim, Germany). LDL cholesterol was calculated with the Friedewald formula [27] adjusted for plasma triglycerides levels [28]. Apolipoprotein (apo) A-I, apo B-100 and lipoprotein(a) [Lp(a)] were measured using a immunonephelometric assay on Array 306 system (Beckman GMI, Inc., Albertville, MN, USA). Quality control of the assays was assured by the Regional Health Authority, as it is compulsory for all hospital clinical laboratories in Spain.

Determination of a composite index of blood lipids and fasting glycaemia

To investigate whether a metabolic composite index was related to aerobic fitness, we decided to compute a continuous score from the following four measurements: triglycerides, HDL cholesterol, LDL cholesterol, and fasting glucose. For each of these variables, a *Z* score was computed as the number of SD units from the sample mean after normalization of the variables, i.e., $Z = ([\text{value} - \text{mean}]/\text{SD})$. The HDL cholesterol *Z* score was multiplied by −1 to indicate higher cardiovascular risk with increasing value. The composite index of blood lipids and fasting glycaemia was the sum of the four *Z* scores. The mean of this continuously distributed metabolic composite index was therefore zero by definition.

Determination of aerobic fitness standards associated with a favourable lipid profile

For aerobic fitness assessment, it was necessary to calculate a normalized score of aerobic fitness (score in the 20-m shuttle run test).

Normalization of the aerobic fitness score

Scores in steps of the 20-m shuttle run test ($n = 2090$) were classified for age (13—14, 14—15, 15—16, 16—17, and 17—18.5 years) and gender (male or female). Because the aerobic physical fitness varies according to age and gender, we standardized the value for age and gender with the use of conversion to a *Z* score. *Z* normalized values (mean 0; SD 1) were obtained for each age group and gender. *Z* normalized value = (value − mean)/SD.

Determination of the presence/absence of healthy lipid profile

In accordance with the American Heart Association [29], based on the NCEP Expert Panel on Blood Cholesterol in Children and Adolescents [30], and based on other studies [31,32], we set undesirable serum total cholesterol, LDL cholesterol and Lp(a) levels higher than 240 mg/dl, 130 mg/dl, and 32 mg/dl, respectively, and HDL cholesterol lower than 35 mg/dl as indicators of lipid cardiovascular risk factors in children and adolescents. We considered the presence of a healthy lipid profile when healthy values were obtained in all of the indicated lipids. Absence of a healthy lipid profile was considered when at least one of the indicated lipid cardiovascular risk factors was detected.

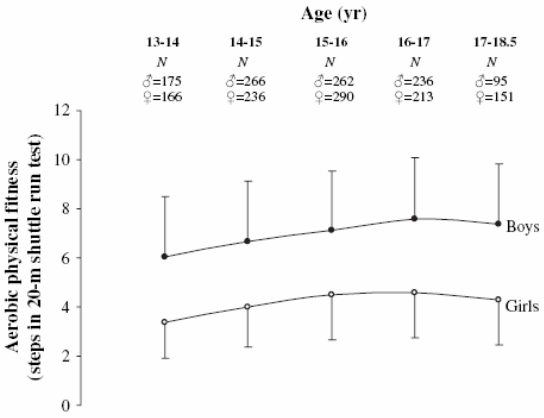

Figure 1 Aerobic fitness scores by gender and age. Data are expressed as mean ± SD.

Calculation of aerobic fitness standards to present a healthy lipid profile

After the dichotomised classification of the lipid profile (presence/absence of healthy lipid profile), receiver operating characteristic (ROC) analysis was performed in order to summarize the potential of age- and gender- normalized aerobic fitness to discriminate between presence or absence of healthy lipid profile [33]. The aerobic fitness standards for presenting a healthy lipid profile were settled defining the best tradeoff between true-positive and false-positive rates [34].

Statistical analysis

Inter-groups differences among gender were assessed by either Student's *t*-test (parametric variables) or Mann—Whitney *U*-test (non-parametric variables). General linear modelling was used to test associations between aerobic fitness, blood lipids, and glycaemia, after adjustment for confounding factors. ROC analysis was used to summarize the potential diagnostic accuracy of aerobic fitness (see above) to discriminate between the presence/absence of a favourable lipid profile.

Table 2 General linear model analysis showing associations between blood lipids and aerobic fitness in adolescents

	Aerobic fitness (quartiles)									
	Boys ($n = 248$)					Girls ($n = 212$)				
	1	2	3	4	*P* for trend	1	2	3	4	*P* for trend
Total cholesterol (mg/dl)	157.6 (33.8)	156.4 (23.5)	155.9 (20.9)	154.4 (23.7)	0.915	166.0 (29.6)	168.6 (29.5)	169.1 (22.3)	169.6 (26.5)	0.903
Triglycerides (mg/dl)	76.1 (37.4)	83.0 (36.4)	65.0 (21.7)	65.3 (27.5)	0.004	69.2 (35.6)	67.4 (23.7)	59.8 (17.7)	60.3 (20.0)	0.191
LDL cholesterol (mg/dl)	94.8 (30.2)	89.2 (20.9)	91.0 (20.8)	84.9 (20.1)	0.202	97.9 (24.4)	102.5 (25.6)	98.1 (19.4)	94.3 (23.7)	0.415
HDL cholesterol (mg/dl)	49.0 (9.1)	50.6 (9.5)	52.7 (9.8)	53.9 (9.3)	0.013	57.2 (10.6)	58.8 (11.4)	60.4 (9.9)	63.4 (14.0)	0.045
Glucose (mg/dl)	96.9 (8.2)	96.1 (8.4)	97.2 (17.8)	95.3 (8.1)	0.798	90.7 (9.1)	95.0 (7.0)	92.7 (7.3)	92.4 (9.7)	0.430
Apo A-I (mg/dl)	111.2 (14.7)	116.4 (18.9)	117.2 (24.5)	121.8 (20.2)	0.028	120.6 (15.3)	120.3 (25.7)	119.2 (22.8)	129.8 (28.4)	0.173
Apo B-100 (mg/dl)	67.0 (17.8)	68.3 (13.4)	68.7 (13.1)	66.5 (14.2)	0.840	71.3 (16.0)	74.6 (16.4)	72.5 (12.4)	68.6 (13.6)	0.261
Lp(a) (mg/dl)	32.9 (38.6)	30.7 (41.3)	31.3 (36.1)	26.2 (28.6)	0.773	29.4 (36.6)	31.0 (40.7)	39.1 (51.4)	26.3 (29.0)	0.573

Results expressed as mean (SD). *P* for trend was calculated after adjustment for age, sexual maturation, and economic status.
LDL refers to low density lipoprotein; HDL, high density lipoprotein; Apo, apolipoprotein; Lp(a), lipoprotein(a).

The level of significance for all analyses was set at $P < 0.05$.

Results

As expected, aerobic physical fitness was significantly higher in males compared with females for all ages ($P < 0.001$) (Fig. 1).

After general linear model analysis adjusted for age, sexual maturation and economic status, aerobic physical fitness was related to triglycerides ($P = 0.004$), HDL cholesterol ($P = 0.013$), and Apo A-I ($P = 0.028$) in male adolescents (Table 2). In females, aerobic fitness was only significantly related to HDL cholesterol ($P = 0.045$) (Table 2).

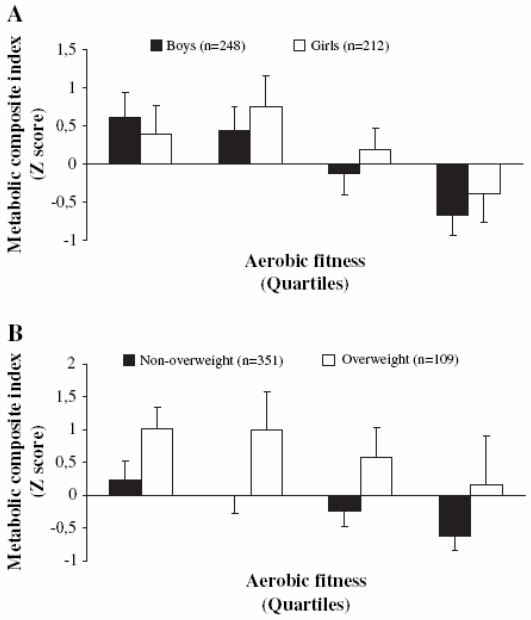

Figure 2 (A) General linear modelling showing associations between a composite index of blood lipids and fasting glycaemia and aerobic fitness in adolescents, after adjustment for age, sexual maturation, and economic status. In males there was a significant association ($P = 0.018$); in females a significant association was found from the 2nd quartile to the 4th quartile of aerobic fitness ($P = 0.045$). Data are expressed as mean ± SEM. (B) Metabolic composite index of blood lipids and fasting glycaemia according to quartiles of aerobic fitness and weight categories, after adjustment for gender, age, sexual maturation, and economic status. The metabolic composite index was significantly higher in overweight adolescents ($P = 0.001$). In both overweight and non-overweight categories, the metabolic composite index was related to aerobic fitness ($P < 0.05$). Data are expressed as mean ± SEM.

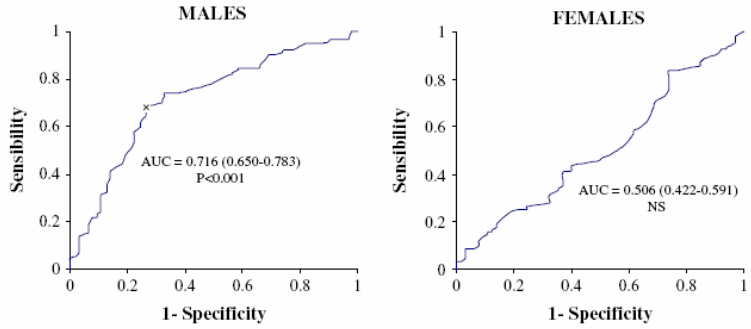

Figure 3 Receiver operating characteristic (ROC) curve summarizing the potential of aerobic fitness to discriminate between the absence/presence of healthy plasma lipid profile in males ($n = 248$) and females ($n = 212$). The cross denotes the best cut-off point to discriminate the absence or presence of healthy plasma lipid profile in males, with a specificity of 0.734. Such a cut-off point was 0.1411 (age-normalized aerobic fitness). AUC, area under the curve.

A composite index of blood lipids and fasting glycaemia was significantly related to aerobic fitness in males ($P = 0.018$), after adjustment for age, sexual maturation and economic status (Fig. 2A). In females, a significant trend was found from the 2nd quartile to the 4th quartile of aerobic fitness ($P = 0.045$) (Fig. 2A). Although the same pattern remained in overweight (including obesity) and non-overweight adolescents after adjustment for gender, age, sexual maturation and economic status ($P < 0.05$, Fig. 2B), the metabolic composite index was significantly higher in overweight (including obesity) adolescents ($P = 0.001$).

ROC analysis showed a significant diagnostic accuracy of age-normalized aerobic fitness to discriminate the presence/absence of healthy plasma lipid profile in males (AUC = 0.716, $P < 0.001$) but not in females (AUC = 0.506, $P = 0.88$) (Fig. 3). The best age-normalized aerobic fitness value capable of discriminating the presence/absence of healthy lipid profile in males was 0.1411 (the cross in Fig. 3 and the dotted line in Fig. 4). Males with age-normalized aerobic fitness values above such a cut-off point presented increased odds (odds ratio 5.91, 95% confidence interval 3.35–10.45, $P < 0.01$) of having a healthy lipid profile than those with aerobic fitness values below 0.1411 (Fig. 4).

Table 3 shows, according to age, the male aerobic fitness needed to present a healthy lipid profile. About 50% of the male study population did not reach such values.

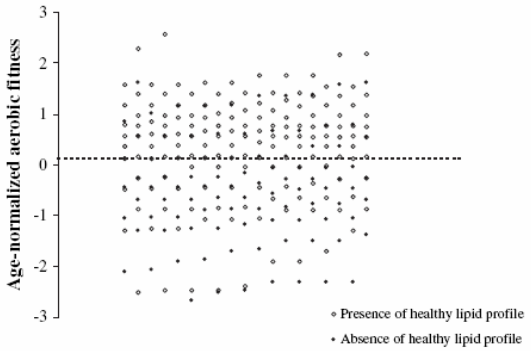

Figure 4 Distribution of the absence or presence of healthy lipid profile in males according to the age-normalized aerobic fitness ($n = 248$). The dotted line represents the best age-normalized aerobic fitness (0.1411) for discriminating the absence/presence of healthy lipid profile. 73.4% of subjects without a healthy lipid profile were below such aerobic standard. The age-normalized aerobic fitness standard for presenting a healthy lipid profile was settled defining the best tradeoff between true-positive and false-positive rates [34].

Discussion

Previous studies in children [16,17,35,36] have shown associations between aerobic physical fitness and blood lipids. We also show here associations between aerobic fitness and single blood lipids, mainly in males. The only association found in girls in our study was to HDL cholesterol levels, but even the first quartile of aerobic physical fitness had mean HDL cholesterol levels of 57.2 ± 10.6 mg/dl, quite acceptable. This discrepancy is also noted by Boreham et al. [17], who revealed significant relationships between three cardiovascular risk factors and fitness in 12 year old boys, whereas only one cardiovascular risk factor and fitness in 12 year old girls.

With respect to the lipid profile and fasting glucose as a whole, we also show here associations between increased aerobic physical fitness and

Table 3 Aerobic fitness standards required to present a healthy lipid cardiovascular profile in males

	Age (years)					True positive rate (%)	False negative rate (%)
	13–14	14–15	15–16	16–17	17–18.5		
Aerobic fitness standard	6.4	7.0	7.5	7.8	7.8	73.4	26.6
Percentage of population above the standard	49.7	51.9	49.2	52.1	51.6		

Aerobic fitness standard values are expressed in steps scored in the 20-m shuttle run test. The aerobic fitness standards for presenting a healthy lipid profile were settled defining the best tradeoff between true-positive and false-positive rates [34].

lower composite index of blood lipids and fasting glycaemia in adolescents, in both overweight and non-overweight adolescents. However, overweight (including obesity) adolescents with high aerobic fitness presented a higher composite index than their non-overweight counterparts with the same aerobic fitness level. This suggests the necessity to improve both aerobic fitness level and weight status in adolescents in order to prevent an unhealthy metabolic profile. This is biologically plausible. Firstly, some studies in children [16,17,35,36] have shown associations between aerobic physical fitness and blood lipids. Secondly, other studies have shown abdominal subcutaneous fat [37,38] and visceral adipose tissue [39,40] as determinants of metabolic atherogenesis risk factors in youth. Finally, we [41] have shown previously that overweight and obesity may induce a chronic low-grade inflammatory state in adolescents, which points out the importance of maintaining an appropriate body weight to avoid obesity-related diseases during adolescence.

Thus, our data suggest that both the promotion of physical activity and the reduction of excessive weight during adolescence may reduce exposure to metabolic risk factors in adolescents, which is in agreement with recent data [42]. According to several longitudinal studies [18,43,44], such a protection during adolescence could be expanded later in life.

In this study we also intended to provide, by using ROC analysis, the minimal values of aerobic physical fitness needed to present a healthy lipid profile in adolescents. Because the association between aerobic physical fitness and blood lipids was weaker in females compared to males, we were able to get the minimal values of aerobic fitness only in males. About 50% of male adolescents did not reach such required aerobic fitness values. Thus, the present aerobic fitness in adolescents seems to be less than acceptable. In this regard, some studies documented a progressive decrease in aerobic fitness among children over the last decades [45,46]. In fact, while in the 1980s children aged 12–14 years had mean of the 20-m shuttle run score of 8.0 ± 1.7 and 6.4 ± 1.5 steps for boys and girls respectively [23], we report updated mean scores of 6.0 ± 2.4 and 3.4 ± 1.5 steps for boys and girls aged 13–14 years. Although aerobic fitness has a strong genetic component [47], and a part of the variability in health-related fitness is not accounted for by physical activity [18,48], such a decrease is probably mediated by a diminution in physical activity among adolescents over recent years.

According to the American Heart Association [29], the decrease in aerobic fitness may be due to a variety of reasons: (1) children tend to walk less and increasingly rely on cars for transportation; (2) sedentary entertainment has increased among adolescents; (3) participation in organized athletics diminishes greatly after middle school. Since physical fitness is very predictable from early years [49], and physical activity during childhood and adolescence is related to adult physical activity [50,51] and may influence the development of cardiovascular risk later in life [44,50], it is of interest to promote physical activity from childhood. In this respect, schools are in a uniquely favourable position to increase aerobic physical activity among their students.

In conclusion, our data suggest that both aerobic fitness and weight management may be necessary for the prevention of metabolic risk in adolescents. In addition, we add the minimal criterion standards of aerobic fitness to present a healthy plasma lipid profile in male adolescents. This is a new tool which should be adopted by schools as ''aerobic fitness standards'' in order to keep a healthy plasma lipid profile and, thus, to prevent metabolic and CVD risk during adolescence and later in life.

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3.2. Anthropometric determinants of a clustering of lipid-related metabolic risk factors in overweight and non-overweight adolescents. Influence of cardiorespiratory fitness.

ABSTRACT

Background/Aims: To explore in adolescents the associations between simple anthropometric variables with a continuously distributed summary score for lipid-related metabolic risk in both overweight and non-overweight adolescents, and to test whether these associations are modified by the level of cardiorespiratory fitness.

Methods: Cardiorespiratory fitness, BMI, skinfold thicknesses, body circumferences, and a continuously distributed clustering of lipid-related metabolic risk (calculated from LDL and HDL cholesterol, triglycerides, and glucose) were measured in 524 adolescents (265 males, 259 females, 15.3+/-1.4 yr) from the cross-sectional multicentric AVENA study. Participants were classified as overweight (including obesity) or non-overweight.

Results: Most anthropometric parameters were univariately related to the continuous lipid-related metabolic risk. However, after multicollinear analysis and generalized linear modelling, suprailiac skinfold thickness in males ($P<0.001$, explained variance 12.2%) and waist-to-height ratio in females ($P<0.001$, explained variance 10.0%) were the best determinants of the continuous metabolic risk score, after adjustment for age, sexual maturation, and economic status. These associations were slightly weakened in overweight males ($P=0.034$) and females ($P=0.087$), and there was no interaction with cardiorespiratory fitness.

Conclusion: Our data emphasize the usefulness of suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk, mainly in non-overweight adolescents and regardless their cardiorespiratory status.

Key words: Adolescence – Metabolic risk – Body composition – Cardiorespiratory fitness

3.2.1. Introduction

Paediatric and adolescent obesity represents an uncontrolled and increasing worldwide epidemic [1-5]. Long-term health complications in overweight children and adolescents include increased rates of metabolic and cardiovascular diseases and mortality [6,7]. In fact, subtle plasma lipid abnormalities predisposing to atherosclerosis are already detected in overweight children and adolescents [8-11]. The continuing upward trends in prevalence of child obesity, and the adverse health implications related to both paediatric and adult obesity, indicate the need for early identification and characterization of the relationship between simple measures of obesity and both cardiovascular and metabolic complications in adolescents [12,13].

Body mass index (BMI) [14,15], although with caution [16], waist circumference [15,17,18], and waist-to-height ratio [17,19] have been proposed as simple anthropometric measures related to metabolic and cardiovascular risk factors in children and adolescents. These previous studies used a categorical definition of cardiovascular risk, which may limit the power to detect an association [20].

In the present study we explored the associations between simple anthropometric variables with a continuously distributed summary score for lipid-related metabolic risk. Since several anthropometric measurements were reported to have less reliability and accuracy in overweight than in lean subjects [21,22], we explored the associations in both overweight (including obesity) and non-overweight adolescents. Interestingly, large-scale epidemiological studies have demonstrated that low cardiorespiratory fitness is a stronger predictor of both cardiovascular and all-cause mortality than other established risk factors [23,24]. Therefore, we also tested whether the association between anthropometric variables and the lipid-related metabolic risk score is modified by the level of cardiorespiratory fitness in adolescents.

3.2.2. Methods

A total of 524 healthy Caucasian adolescents from five different geographic locations from Spain took part in the study. Complete clinical, anthropometric, and cardiorespiratory fitness data were available for all participants. The subjects were all participants in the AVENA Study [25,26], a population-based cross-sectional multicentric study of the aetiology and pathogenesis of obesity and related metabolic disorders. Parents and school supervisors were informed by a letter about the nature and purpose of the study and they gave their

written informed consent. The study protocol was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki, and approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

Cardiorespiratory fitness

Cardiorespiratory fitness was measured by the progressive 20-m shuttle run test [27], which has been validated for use in children [28]. This test required subjects to run back and forth between two lines set 20 m apart. Running pace was determined by audio signals, emitted from a pre-recorded cassette tape, the initial velocity being 8.5 km·h-1, and increasing by 0.5 km·h-1 every minute (step). The tape used was calibrated over 1 min duration and the tape machine was checked for accuracy prior to each test. Subjects were instructed to run in a straight line, to pivot upon completing a shuttle and to pace themselves in accordance to the time intervals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on two consecutive occasions. Scoring was by steps completed (precision of 0.5 steps). A constant level of encouragement was given to participants throughout the test. Subjects were instructed to abstain from strenuous exercise over 48 h preceding the test.

Anthropometric assessment

For anthropometric measurements, subjects were barefoot and in their underwear. Weight was measured with a Seca scale (precision of 50 g), and height with incorporated stadiometer to the scale (precision of 1 mm). Biceps, triceps, subscapular, suprailiac, thigh, and calf skinfold thicknesses were measured with a Holtain lipocaliper (range 0–40 mm, precision of 0.1 mm). Arm, thigh, waist and hip circumferences were measured with an inelastic tape (precision of 1 mm). Harmonization and standardization of anthropometric measurements used to assess body composition within the AVENA multicenter study was strictly controlled and has been previously published elsewhere [5,26]. To establish the overweight (including obesity) and non-overweight categories, we used the gender- and age-adjusted cut-off points provided by Cole et al. [14].

Blood sampling

Blood collection was carried out between 8:00 and 9:00 a.m., and after an 8-h overnight fast. The subjects were selected randomly (according to age, sex, geographical location, and economic status) and were instructed to abstain from alcoholic beverages for at least two weeks before sampling and to refrain from vigorous exercise during the 48 h preceding

blood collection. Within one hour after collection, blood was centrifuged and aliquots of sera were sent refrigerated to a central laboratory (Clinical Biochemistry Service, Granada University Hospital) where all the clinical chemistry tests were performed within 24 hours after collection. Plasma glucose, triglycerides and high density lipoprotein (HDL) cholesterol were measured by enzymatic assay using a Hitachi 911 Analyzer (Roche Diagnostics, Indianapolis, Ind, USA). For the HDL cholesterol assay, precipitation was done using reagents provided by Boehringer (Ingelheim, Germany). LDL cholesterol was calculated with the Friedewald formula [29] adjusted for plasma triglycerides levels [30]. Quality control of the assays was assured by the Regional Health Authority, as compulsory for all hospital clinical laboratories in Spain.

Lipid-related metabolic risk score

To investigate whether the clustering of lipid-related metabolic risk factors was related to body composition variables, we decided to compute a continuous risk score from the following four measurements: triglycerides, HDL cholesterol, LDL cholesterol, and glucose. For each of these variables, a Z score was computed as the number of SD units from the sample mean after normalization of the variables, i.e., Z = ([value - mean]/SD). The HDL cholesterol Z score was multiplied by -1 to indicate higher cardiovascular risk with increasing value. The lipid-related metabolic risk score was the sum of the four Z scores. The mean of this continuously distributed lipid-related metabolic risk score is therefore zero by definition.

Statistical analysis

Inter-groups differences among gender were assessed by either Student's test (parametric variables) or Mann Whitney U test (non-parametric variables). Multicollinear analysis and generalized linear modelling were used for assessing the relationship between body composition variables (BMI, waist-to-hip ratio, skinfold thicknesses and circumferences) and the lipid-related metabolic risk score. To test the interaction between anthropometric variables and cardiorespiratory fitness on the metabolic risk score, further linear modelling was undertaken. The level of significance for all analyses was set at $P<0.05$.

3.2.3. Results

The descriptive characteristics of participants are shown in Table 1. Male adolescents were significantly taller and heavier and had higher waist circumference and cardiorespiratory fitness than females ($P<0.001$). Girls presented lower plasma glucose and triglycerides

concentration (P<0.01), and higher plasma HDL (P<0.001) and LDL cholesterol levels (P<0.01).

In boys and girls, the lipid-related metabolic risk score was univariately correlated with suprailiac, biceps, triceps, subscapular, thigh and calf thicknesses (P<0.01), BMI, arm and thigh circumferences (P<0.01), and with waist-to-hip ratio (P<0.05). Additionally, cardiorespiratory fitness was inversely correlated with the metabolic risk score in boys (P=0.001), but not in girls (P=0.198).

In order to calculate the metabolic risk score's variance accounted for by each anthropometric measurement, multicollinear analysis and generalized linear modelling were carried out after adjustment for age, sexual maturation, and economic status. In males, the suprailiac skinfold thickness accounted for the highest amount of metabolic risk score's variance (12.2%, P<0.001), and had a good level of tolerance (14.1%, i.e., 14.1% of suprailiac skinfold thickness' variance was not accounted for by the remaining anthropometric variables). In females, waist-to-height ratio accounted for the highest amount of metabolic risk score's variance (10.0%, P<0.001), with 7.6% of tolerance. (Figure 1). After stepwise multiple regression analysis the previous figures were confirmed, with suprailiac skinfold thickness in males (P<0.001) and waist-to-height ratio in females (P<0.001) as the best estimators of the lipid-related metabolic risk score.

After stratifying the sample into overweight (including obesity) and non-overweight, and by using the same analyses, suprailiac skinfold thickness was the best estimator of the metabolic risk score in both overweight (P=0.034) and non-overweight (P=0.001) male adolescents (Table 2). Biceps and triceps skinfold thicknesses, and arm and thigh circumferences were significantly related to the metabolic risk score in non-overweight, but not in overweight males. In females, waist-to-height ratio was the best estimator of the metabolic risk score in both non-overweight (P=0.020) and overweight (P=0.087) categories (Table 3).

To determine whether the associations between the lipid-related metabolic risk score with suprailiac skinfold thickness in males and waist-to-height ratio in females were affected by the level of cardiorespiratory fitness, generalized linear modelling was carried out. Data were stratified above and below the mean for cardiorespiratory fitness. In addition, data were also stratified by tertiles of suprailiac skinfold thickness in males, and by tertiles of waist-to-height ratio in females. Suprailiac skinfold thickness (P=0.008, in males) and waist-to-height ratio (P=0.005, in females) were significantly related to the lipid-related metabolic risk score after adjustment for BMI, age, tanner stage, and socioeconomic status (Figure 2). These associations were significant after additional adjustment for

cardiorespiratory fitness (P=0.034 in males, P=0.002 in females). No significant interaction was found between such anthropometric measurements and cardiorespiratory fitness in relation to the lipid-related metabolic risk score.

3.2.4. Discussion

Our data show that suprailiac skinfold thickness (in males) and waist-to-height ratio (in females) are the best single estimators of a continuously distributed lipid-related metabolic risk score in a population sample of adolescents from Spain. Both associations were slightly weakened in overweight adolescents, and there was no interaction with cardiorespiratory fitness.

Previous studies [14,15] have proposed BMI as a simple anthropometric measure related to cardiovascular risk factors in children and adolescents. Our data, after categorising into overweight and non-overweight subjects, suggest a limited usefulness of BMI to estimate a clustering of metabolic traits in children and adolescents. In consonance with this, other studies highlight important shortcomings of the BMI measurement to provide information on body fat distribution [31-33] thereby masking true obesity-related metabolic and cardiovascular risk in children [17]. These weaker associations between BMI and cardiovascular risk among children and adolescents may be attributable, in part, to the asynchronous changes that occur in the levels of fat mass and fat-free mass during growth. In fact, it has been shown that BMI increases in adolescents from both sexes are primarily determined by increases in fat-free mass rather than in body fat compartment [16,34].

Our data also demonstrate a less usefulness of waist-to-hip ratio for cardiovascular risk screening in adolescents, questioning the clinical relevance of waist-to-hip ratio as a diagnostic test in adolescents. This is in agreement with recent studies [35,36], and it may be explained by the fact that waist-to-hip ratio has previously been shown to be less valuable for central fatness screening not only in children and adolescents [37,38], but also in adults [39].

In other studies [15,17,18,40] waist circumference has been shown to be a simple anthropometric measure related to cardiovascular and metabolic risk factors in children and adolescents. Although in this study waist circumference was significantly related to the metabolic risk score in both males and females before taking into account weight status, we also found a very low tolerance for waist circumference in both sexes (<1%). Tolerance is the proportion of a variable's variance not accounted for by other independent variables in

the model. Therefore, tolerance is used to determine how much the independent variables (waist circumference and the remaining anthropometric variables) are linearly related to one another (multicollinear). A variable with very low tolerance contributes little information to a model, and can cause computational problems. After dividing waist circumference by height, not only tolerance was acutely increased, but also the power of the variable (waist-to-height ratio) to estimate the lipid-related metabolic risk score. In fact, after multicollinear and linear modelling, suprailiac skinfold thickness (in males) and waist-to-height ratio (in females) were the best estimators of the lipid-related metabolic risk score in adolescents. These findings are biologically plausible. Firstly, we [41] and others [42] found skinfold thickness measurements and waist-to-height ratio to be estimators of the visceral (intra-abdominal) mass of adipose tissue along with the subcutaneous truncal fat mass. Secondly, other studies have shown abdominal subcutaneous fat [43,44] and visceral adipose tissue [45,46] as determinant of metabolic risk factors in youth. Moreover, since our data were obtained after adjustment for confounding factors, our observations are unlikely to be explained by chance, bias, or confounding. In addition, the lipid-related metabolic risk *Z* score, which we used to define a metabolic risk clustering on a continuous scale, is statistically more sensitive and less error prone by comparison to other approaches [20,47,48].

Several anthropometric measurements (waist circumference and suprailiac skinfold thickness among them) were reported to have less reliability and accuracy in overweight than in lean subjects [21,22]. We showed suprailiac skinfold thickness (in males) and waist-to-height ratio (in females) as the best estimators of lipid-related metabolic risk mainly in non-overweight adolescents, although such variables also remained as the best estimators in over-weight adolescents. These results are probably due to the high reliability we got in both waist circumference and suprailiac skinfold thickness in the AVENA study [26]. Of note, the intra- and inter-observer technical error of measurements for skinfold thickness and waist circumference in our survey were lower than the reference values proposed by Ulijazsek and Lourie [49] and Ulijazsek and Kerr [50]. The slightly weakened associations of such anthropometric variables with the metabolic risk score in the overweight category may be due to its small sample size by comparison to the non-overweight category.

Finally, we also tested whether the associations between suprailiac skinfold thickness and waist-to-height ratio with an overall metabolic risk score is modified by the level of cardiorespiratory fitness in adolescents. We did not get any interaction between cardiorespiratory fitness and such anthropometric variables in estimating the lipid-related

metabolic risk score, and our results suggest that such anthropometric variables are independently related to the lipid-related metabolic risk score. Although the cross-sectional nature of our study limits inference about the direction of causality, it is biologically plausible that a reduction in suprailiac skinfold thickness in males and waist-to-height ratio in females improves the lipid-related metabolic risk profile in adolescents. Firstable, subcutaneous truncal fat is an independent estimator of metabolic traits [43,44]. Secondly, the well-known association between cardiorespiratory fitness and these risk factors may be mediated by the levels of abdominal subcutaneous fat [44]. Whether improvements in suprailiac skinfold thickness or waist-to-height ratio may cause improvements in the cardiovascular risk profile in adolescents, independently of changes in cardiorespiratory fitness, remains to be elucidated in further studies.

In conclusion, after multicollinear analysis and linear modelling, suprailiac skinfold thickness (in males) and waist-to-height ratio (in females) were the best simple anthropometric estimators of a clustering of lipid-related metabolic traits in a population of adolescents from Spain, mainly in non-overweight individuals and regardless their cardiorespiratory status. These simple measurements should be used for early identification and characterization of metabolic complications in adolescents.

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Table 1. Descriptive characteristics of participants

	Boys (n=265)	**Girls** (n=259)
Age (years)	15.3 ± 1.3	15.4 ± 1.4
Height (cm)	170.9 ± 7.7	161.4 ± 6.4 •••
Weight (kg)	64.5 ± 12.9	56.2 ± 10.9 •••
BMI (kg/m^2)	21.9 ± 3.8	21.5 ± 3.5
Waist circumference (cm)	77.0 ± 9.2	70.8 ± 8.3 •••
Cardiorespiratory fitness (steps)	7.1 ± 2.7	4.1 ± 1.8 •••
Triglycerides (mg/dl)	71.8 ± 31.9	63.4 ± 24.7 ••
HDL cholesterol (mg/dl)	51.6 ± 9.7	59.9 ± 11.9 •••
LDL cholesterol (mg/dl)	90.2 ± 23.1	95.4 ± 23.4 ••
Glucose (mg/dl)	96.4 ± 11.3	91.9 ± 8.9 •••

Data are means ± SD. Student's test (parametric variables) or Mann Whitney U test (non-parametric variables) for differences between sexes: •• P<0.01, ••• P<0.001. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2. Generalized linear modelling showing associations between several anthropometric variables and the lipid-related metabolic risk z-score, in non-overweight and overweight male adolescents.

	Non-overweight (n=192)			**Overweight** (n=73)		
	β coefficient		*P*	β coefficient		*P*
Skinfold thickness	1T	2T		1T	2T	
Biceps	-1.038 (0.421)	-0.346 (0.446)	**0.044**	0.774 (0.889)	0.037 (0.825)	0.589
Triceps	-0.748 (0.443)	0.213 (0.451)	**0.049**	0.296 (0.923)	-0.228 (0.780)	0.829
Suprailiac	-1.415 (0.395)	-0.248 (0.435)	**0.001**	-1.190 (0.830)	-0.327 (0.817)	**0.034**
Subscapular	-0.911 (0.420)	-0.227 (0.428)	0.085	-1.787 (0.861)	-0.616 (0.757)	0.120
Subscapular/Triceps	-0.063 (0.467)	0.004 (0.424)	0.987	-1.127 (0.801)	0.026 (0.754)	0.313
Thigh	-0.812 (0.442)	-0.699 (0.450)	0.156	0.330 (0.834)	-0.151 (0.834)	0.816
Calf	-0.827 (0.448)	-0.526 (0.471)	0.185	-0.184 (0.869)	0.085 (0.841)	0.939
Circumferences						
Arm	-1.627 (0.431)	-0.778 (0.399)	**0.001**	-1.226 (0.837)	0.017 (0.802)	0.256
Waist	-0.871 (0.474)	-0.330 (0.413)	0.185	0.234 (0.872)	0.340 (0.800)	0.913
Hip	-0.687 (0.472)	-0.819 (0.424)	0.142	0.322 (0.838)	0.522 (0.877)	0.838
Thigh	-1.075 (0.456)	-0.646 (0.393)	**0.050**	-0.047 (0.833)	-0.616 (0.806)	0.697
Other indices						
BMI	-1.019 (0.438)	-0.509 (0.423)	0.07	-0.057 (0.893)	-0.261 (0.851)	0.949
W:Hp ratio	0.353 (0.438)	0.298 (0.439)	0.693	-0.247 (0.876)	0.668 (0.782)	0.493
W:Ht ratio	-0.875 (0.412)	-0.165 (0.426)	0.091	0.157 (0.776)	-0.251 (0.850)	0.888

Analyses were performed after adjustment for age, sexual maturation and socioeconomic status. 1T refers to 1st tertile; 2T, 2nd tertile; BMI, body mass index; W, waist; Hp, hip; Ht, height.

Table 3. Generalized linear modelling showing associations between several anthropometric variables and the lipid-related metabolic risk z-score, in non-overweight and overweight female adolescents.

	Non-overweight (n=212)			Overweight (n=47)		
	β coefficient		*P*	β coefficient		*P*
Skinfold thickness	1T	2T		1T	2T	
Biceps	-0.806 (0.414)	-0.634 (0.423)	0.133	0.565 (0.819)	0.735 (0.833)	0.330
Triceps	-0.649 (0.431)	-0.605 (0.451)	0.280	-1.463 (0.858)	-0.317 (0.814)	0.221
Suprailiac	-0.861 (0.427)	-1.158 (0.427)	**0.024**	-0.383 (0.855)	-0.318 (0.830)	0.890
Subscapular	-0.416 (0.411)	-0.468 (0.435)	0.493	-1.251 (0.826)	0.043 (0.871)	0.278
Subscapular/Triceps	-0.188 (0.404)	0.235 (0.424)	0.633	-0.750 (0.941)	-0.093 (0.767)	0.714
Thigh	-0.084 (0.452)	-0.407 (0.448)	0.600	-1.238 (0.844)	-0.607 (0.894)	0.354
Calf	-0.253 (0.447)	-0.015 (0.445)	0.796	-0.452 (0.878)	0.609 (0.877)	0.474
Circumferences						
Arm	-0.545 (0.415)	0.124 (0.426)	0.267	-0.756 (0.988)	-0.614 (0.887)	0.697
Waist	-0.726 (0.430)	-0.674 (0.428)	0.168	-1.368 (0.760)	-0.673 (0.834)	0.210
Hip	-0.566 (0.380)	-0.747 (0.394)	0.145	-0.372 (0.852)	-0.334 (0.897)	0.893
Thigh	-0.239 (0.382)	-0.352 (0.382)	0.642	-0.039 (0.853)	-0.166 (0.846)	0.980
Other indices						
BMI	-0.708 (0.557)	-0.789 (0.387)	0.122	-1.532 (0.812)	-0.698 (0.810)	0.186
W:Hp ratio	-0.510 (0.388)	-0.183 (0.378)	0.411	-1.212 (0.984)	-0.437 (0.913)	0.427
W:Ht ratio	-0.837 (0.378)	-0.596 (0.385)	**0.020**	-1.187 (0.695)	-0.234 (0.816)	0.087

Analyses were performed after adjustment for age, sexual maturation and socioeconomic status. 1T refers to 1st tertile; 2T, 2nd tertile; BMI, body mass index; W, waist; Hp, hip; Ht, height.

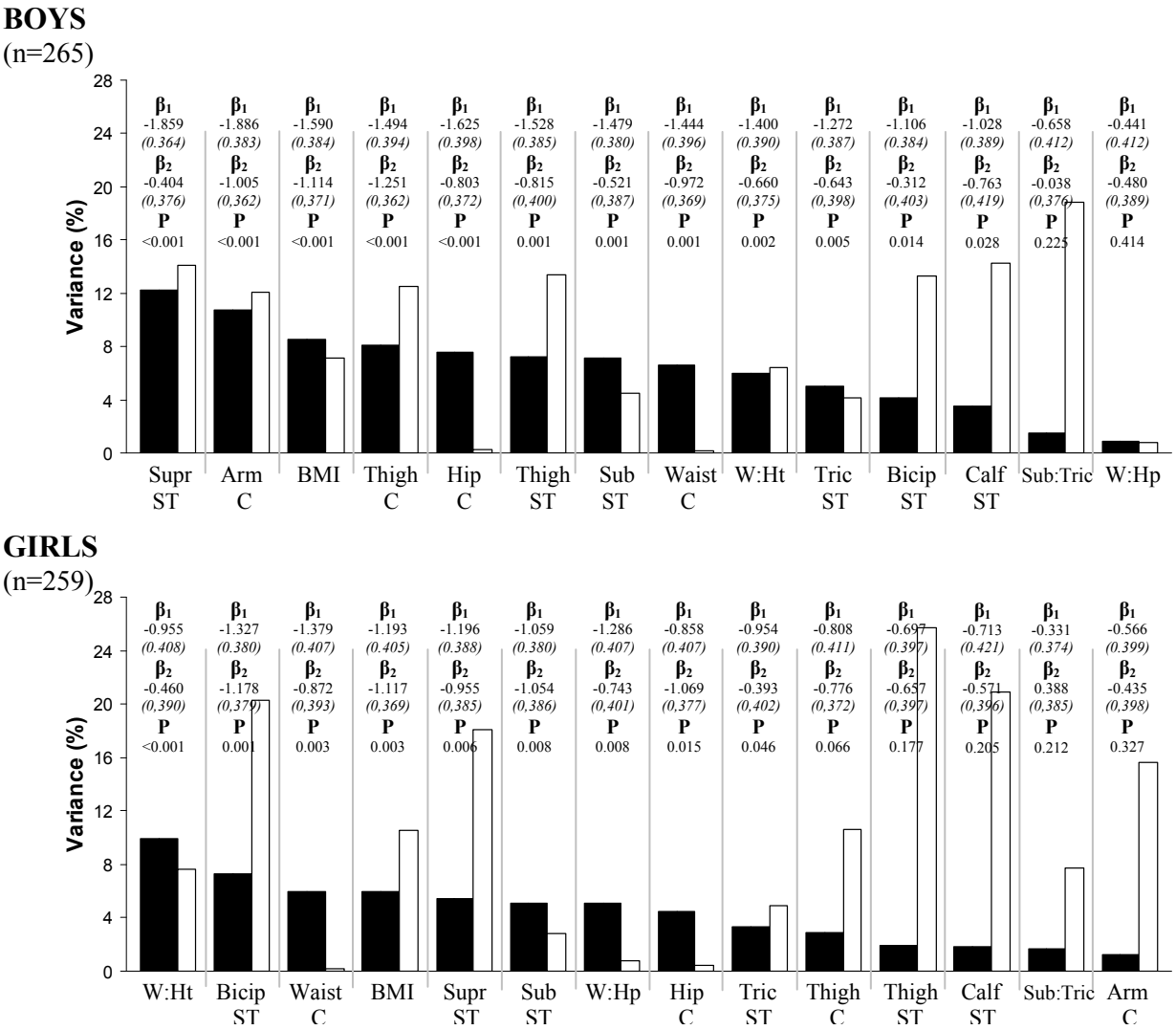

Figure 1. Multicollinear analysis and linear modelling showing associations between several anthropometric measures and the lipid-related metabolic risk z-score in male and female adolescents. The analyses were performed after adjustment for age, sexual maturation, and economic status. Black columns refer to the lipid-related atherogenesis risk score's variance explained by different anthropometric variables. White columns refer to the proportion of every anthropometric measurement's variance not accounted by the other anthropometric variables (tolerance).

β_1 refers to β coefficient for 1st tertile, β_2 refers to β coefficient for 2nd tertile. P denotes the level of significance for a trend among tertiles in every anthropometric measure.

W:Ht, waist-to-height ratio; W:Hp, waist-to-hip ratio; Sub:Tric, subscapular ST / tricipital ST ratio; Supr, suprailiac; Sub, subscapular; Bicip, bicipital; Tric, tricipital; BMI, body mass index; ST, skinfold thickness; C, circumference.

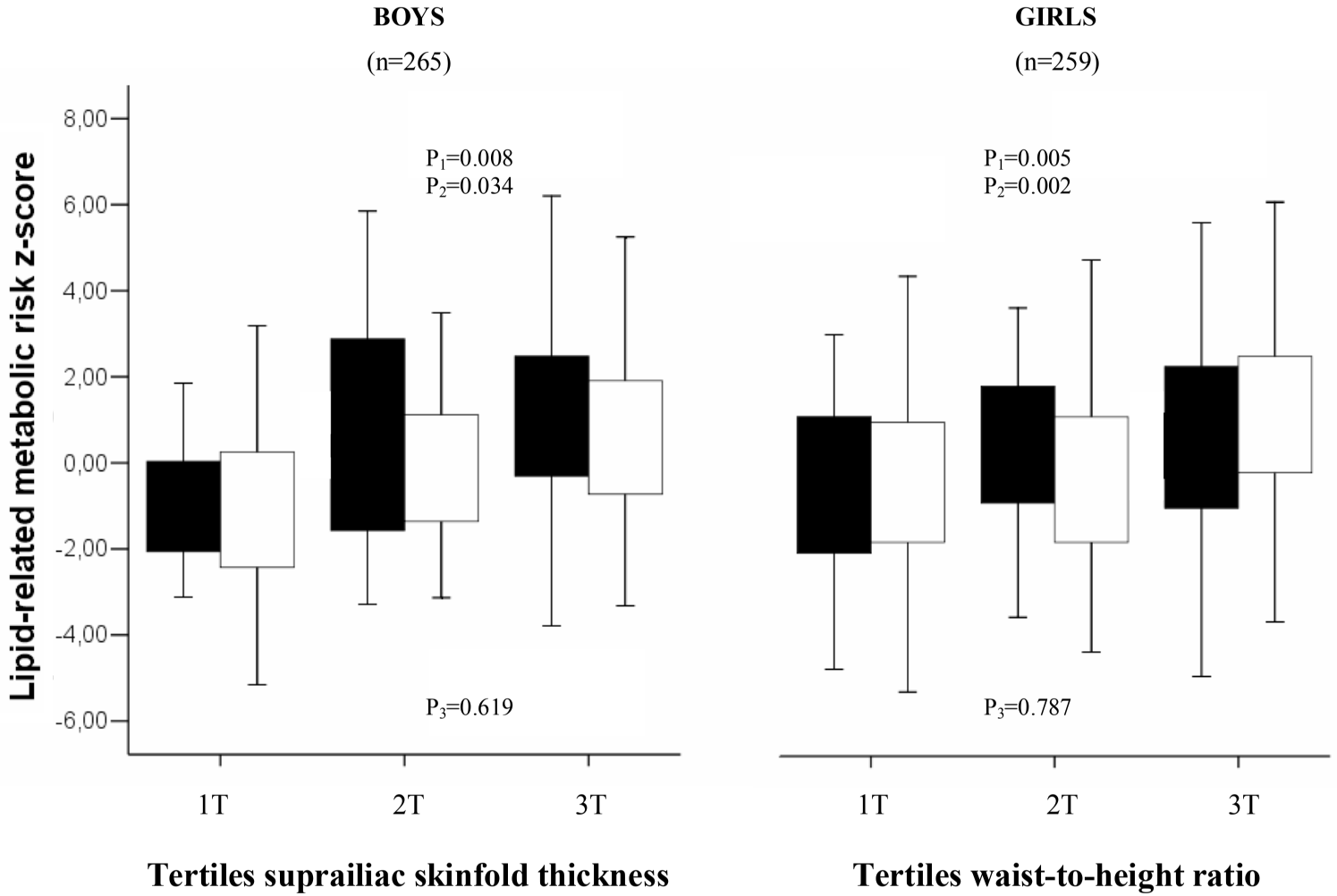

Figure 2. Associations of suprailiac skinfold thickness (boys) or waist-to-height ratio (girls) with the lipid-related metabolic risk z-score for low (□) or high (■) cardiorespiratory fitness. Boxes represent interquartile ranges, and whiskers extreme values that are not outliers. P_1 denotes the level of significance of the association (by using generalized linear modelling) after adjustment for BMI, age, tanner stage, and socioeconomic status. P_2 was calculated after additional adjustment for cardiorespiratory fitness. P_3 denotes the level of significance of the interaction (suprailiac skinfold thickness or waist-to-height ratio) x cardiorespiratory fitness.

4.

Aerobic fitness and common apolipoprotein gene variants on metabolic and immune outcomes

In the previous section of this PhD Thesis we underscore the importance of aerobic physical fitness and body composition to keep a healthy lipid-related metabolic profile in adolescents. However, interrelations with common apolipoprotein gene variants or immune outcomes (well known mediators of lipid disturbances and cardiovascular risk) were not studied. We study these interrelations in this section.

What is already known on this topic

Common apolipoprotein variants of *APOC3* and *APOE* genes influence lipid-related metabolic traits. However, to our knowledge there are no studies analysing possible interactions between these genes and physical fitness in relation to lipid-related metabolic traits in adolescents.

Several studies in adolescents have assessed complex relationships between fitness, fatness and inflammation. However, there are no studies in this population analysing possible interactions with common apolipoprotein gene variants.

What this PhD Thesis adds

We provide genotype-dependent aerobic physical fitness levels associated to a favourable lipid-related metabolic profile in adolescents.

We suggest that cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents, regardless physical fitness or weight status.

This section comprises two studies analysing interrelations of aerobic physical fitness, anthropometric measurements and common apolipoprotein gene variants in relation to lipid- and immune-related metabolic traits:

Niveles mínimos de capacidad aeróbica asociados a bajo riesgo lipídico-metabólico en adolescentes en función del perfil genético de apoproteínas

[Aerobic fitness standards related to low metabolic risk in adolescents according to apolipoprotein gene variants]

MESA JL, GONZÁLEZ-LAMUÑO D, RUIZ JR, ORTEGA FB, CARREÑO F, GUTIÉRREZ A, CASTILLO MJ, GARCÍA-FUENTES M. *Rev Esp Cardiol* 2006 (*submitted*).

Cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents

MESA JL, WÄRNBERG J, RUIZ JR, ORTEGA F, GUTIÉRREZ A, MARCOS A, CASTILLO MJ. (Manuscript).

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4.1. [Aerobic fitness standards related to low metabolic risk in adolescents according to apolipoprotein gene variants]

RESUMEN

Introducción y Objetivos: Determinar los niveles mínimos de capacidad aeróbica asociados a un bajo riesgo lipídico-metabólico en adolescentes, considerando polimorfismos de los genes *APOC3* y *APOE*.

Métodos: Los sujetos utilizados pertenecieron al estudio transversal multicéntrico AVENA. La capacidad aeróbica se determinó en 2090 adolescentes mediante el test de Course Navette. Se calculó un índice de riesgo lipídico-metabólico (definido por los niveles plasmáticos de glucemia, triglicéridos, cLDL, cHDL e índice de masa corporal) en 470 de los anteriores sujetos. Finalmente, 420 de los anteriores sujetos se caracterizaron para polimorfismos comunes de los genes *APOC3* (alelos S1 y S2) y *APOE* (alelos ε2, ε3 y ε4).

Resultados: La capacidad aeróbica se relacionó con el índice de riesgo lipídico-metabólico tanto en niños ($p<0.001$) como en niñas ($p=0.012$), tras ajustar por edad y maduración sexual. Al tener en cuenta variantes genéticas, y tras ajustar por género, edad y maduración sexual, diferentes relaciones ($p<0.05$) entre capacidad aeróbica y nivel de riesgo lipídico-metabólico fueron obtenidas para cada variante genética, excepto para los portadores del alelo ε2 de *APOE*. Los portadores del alelo S2 del gen *APOC3* o del alelo ε4 de *APOE* (en total 33% de la población analizada) debían tener mayor capacidad aeróbica para presentar el mismo índice de riesgo lipídico-metabólico que los portadores de los genes *APOC3* S1/S1 o *APOE* ε3/ε3.

Conclusiones: El presente estudio sugiere que la capacidad aeróbica necesaria en adolescentes para presentar un bajo riesgo lipídico-metabólico debe de ser individualizada en función de aspectos genéticos.

Palabras clave: Ejercicio – Lípidos – Genética

SUMMARY

Introduction and aims: This study aimed to set minimal aerobic standards associated to low lipid-metabolic risk in adolescents, according to common variants in *APOC3* and *APOE* genes.

Methods: All participants in this study were recruited from the multicenter cross-sectional AVENA study. Cardio-respiratory fitness was determined in 2090 adolescents by using the Course-Navette test. A lipid-metabolic risk score was computed in 470 subjects from glycaemia, plasma triglycerides, LDLc, HDLc, and body mass index. Finally, 420 subjects were genotyped for common polymorphisms in *APOC3* (S1/S2) and *APOE* (ε2, ε3, ε4) genes.

Results: After adjustment for age and sexual maturation, cardio-respiratory fitness was related to the lipid-metabolic risk score in boys ($p<0.001$) and girls ($p=0.012$). After adjustment for gender, age, and sexual maturation, genotype-dependent associations between cardio-respiratory fitness and lipid-metabolic risk were found for all genetic variants ($p<0.05$), except for the *APOE* ε2 carriers. *APOC3* S2 or *APOE* ε4 carriers (33% of population analysed) should keep a higher cardio-respiratory fitness to present the same lipid-metabolic status than carriers of wild type genotypes *APOC3* S1/S1 or *APOE* ε3/ε3.

Conclusions: Our data suggest that the required cardio-respiratory fitness levels to present a low lipid-metabolic risk in adolescents should be set according to common apolipoprotein genetic variants.

Key words: Exercise – Lipids – Genetics

4.1.1. Introducción

La enfermedad cardiovascular es la primera causa de muerte en países desarrollados.[1] Aunque las manifestaciones clínicas de la enfermedad cardiovascular ocurren durante la edad adulta, los procesos fisiológicos que la originan comienzan durante la infancia y adolescencia.[2,3] Es por ello que la prevención de enfermedades metabólicas y cardiovasculares debe empezar durante la infancia y la adolescencia.[4]

El ejercicio físico aeróbico, practicado regularmente, está considerado como una de las mejores estrategias para prevenir el desarrollo de enfermedad cardiovascular. De hecho, un bajo nivel de capacidad aeróbica es un factor de riesgo y potente predictor de morbilidad y mortalidad por todas las causas en general y por causas cardiovasculares en particular.[5,6] Debido a que los procesos fisiológicos desencadenantes de enfermedades metabólicas y cardiovasculares futuras comienzan en la infancia y adolescencia, el ejercicio físico aeróbico como medida preventiva e incluso terapéutica debería empezar a realizarse durante dichas etapas. La celeridad en la aplicación de programas globales de ejercicio físico para la población infantil y adolescente urge aún más tras haberse constatado durante los últimos años un incremento en la incidencia de obesidad y riesgo de enfermedades cardiovasculares y metabólicas en dichas poblaciones.[7-9] España no es ajena a esta tendencia, siendo actualmente uno de los países europeos con mayor prevalencia de sobrepeso y obesidad en adolescentes[10] y el segundo de Europa con mayor incidencia de sobrepeso infantil.[11] Esto implica de manera ineludible un mayor riesgo metabólico-cardiovascular y mayor incidencia de dichas enfermedades en un futuro próximo.

Nuestro grupo de investigación ha caracterizado recientemente la capacidad aeróbica en la población adolescente española[12] y ha propuesto los niveles de capacidad aeróbica requeridos en adolescentes para presentar bajo riesgo lipídico-metabólico (Mesa et al., datos no publicados). Estudios recientes han mostrado que factores ambientales (actividad física y nutrición) interactúan con factores genéticos (principalmente los genes *APOC3* y *APOE*) en relación al riesgo metabólico y cardiovascular en adultos.[13,14] Concretamente, dichas interacciones son producidas con las isoformas ε2, ε3 y ε4 del gen *APOE* y el polimorfismo SstI del gen *APOC3* (que resulta de la transversión entre una base de guanina y otra de citosina en la región no codificadora del gen *APOC3*, originando los alelos S1 y S2). Estos estudios sugieren que el efecto de dichas variantes genéticas puede ser modulado por factores externos, tales como nutrición y actividad física. Dicha modulación podría venir ocurriendo desde etapas tempranas de la vida. En este sentido, más importante que la actividad física sería su repercusión sobre el estado de forma física, tal y como se refleja con la capacidad aeróbica.[12]

En la actualidad no existen estudios analizando interacciones genéticas con capacidad aeróbica en la etapa de la adolescencia. Dada la importancia de la etapa adolescente en cuanto a riesgo cardiovascular futuro, una precisa y correcta individualización (en función del genotipo individual) de la prescripción de ejercicio físico en dicha etapa es crucial. Por tanto, los objetivos de este trabajo de investigación son 1) determinar las interacciones existentes entre capacidad aeróbica y los genes *APOC3* y *APOE* en adolescentes en relación al riesgo lipídico-metabólico y 2) proponer los niveles requeridos de capacidad aeróbica en adolescentes, en función de variables genéticas, para presentar un bajo nivel de riesgo lipídico-metabólico.

4.1.2. Métodos

Sujetos

El presente trabajo de investigación fue realizado bajo el contexto del estudio AVENA, un estudio multicéntrico transversal realizado en cinco diferentes áreas geográficas de España (Granada, Murcia, Madrid, Zaragoza y Santander) con objeto de evaluar la patogénesis de la obesidad y enfermedades metabólico-cardiovasculares asociadas en adolescentes. La metodología completa del estudio puede ser consultada en previas publicaciones.[15,16] Brevemente, 2851 adolescentes españoles (1354 niños y 1497 niñas) de entre 13 y 18 años fueron seleccionados mediante muestreo polietápico, aleatorio y estratificado por: 1) procedencia (Granada, Madrid, Santander, Zaragoza y Murcia); 2) condiciones socio-económicas (en base a la localización del centro educativo, información aportada por las diferentes Consejerías de Educación autonómicas); 3) sexo y edad. Se establecieron los siguientes criterios de exclusión: diagnóstico clínico de diabetes, embarazo, abuso de alcohol o drogas y en general patologías que no estén relacionadas directamente con la nutrición. La exclusión efectiva del estudio se aplicó a posteriori, sin conocimiento por parte de los alumnos. Los padres y supervisores de los alumnos fueron debidamente informados sobre la naturaleza y propósito del estudio, dando su consentimiento informado. El protocolo del estudio fue realizado conforme a los estándares éticos de la Declaración de Helsinki de 1975 (revisada en Edinburgh en el año 2000), y fue aprobado por el Comité de Ética del Hospital Universitario Marqués de Valdecilla (Santander, España).

Medición de la capacidad aeróbica

La capacidad aeróbica fue medida mediante el test de Course Navette[17] en 2090 adolescentes del estudio AVENA (Tabla 1). Su validez y fiabilidad para su uso con niños y

adolescentes ha sido ampliamente contrastada.[18-21] Desde entonces, el test de Course Navette ha sido ampliamente utilizado en colegios e institutos de todo el mundo para evaluar la capacidad aeróbica en niños y adolescentes. El test consistió en recorrer de forma incremental, ida y vuelta entre dos líneas separadas entre sí 20 m. El ritmo de carrera fue marcado por señales acústicas, comenzando a 8,5 km/h, y aumentando 0,5 km/h cada minuto. El test finalizaba cuando el sujeto era incapaz de seguir el ritmo marcado durante dos señales acústicas consecutivas. La puntuación fue dada en paliers (cada palier equivale a un minuto del test). Los sujetos fueron animados constantemente durante el test, y fueron instruidos para abstenerse de realizar ejercicio físico extenuante durante las 48 h previas al mismo.

Análisis bioquímico

Con objeto de analizar el perfil lipídico de los adolescentes, y analizar sus posibles relaciones con la capacidad aeróbica, los sujetos se sometieron a extracciones sanguíneas al menos 3 días antes de la realización del test de Course Navette. En total, 470 muestras sanguíneas fueron obtenidas de adolescentes que ejecutaron el test de Course Navette correctamente. Las extracciones sanguíneas fueron realizadas en ayunas, entre las 8:00 y las 9:00 a.m. Los sujetos fueron seleccionados aleatoriamente (según edad, género, localización geográfica y nivel económico), y fueron avisados de no ingerir alcohol ni realizar ejercicio físico extenuante las 48 h previas a la extracción sanguínea. Las muestras sanguíneas fueron inmediatamente centrifugadas y el suero fue enviado refrigerado al laboratorio central del estudio (Servicio de Bioquímica Clínica, Hospital Universitario Virgen de las Nieves, Granada), donde todos los análisis bioquímicos fueron realizados.

Los niveles de glucosa, triglicéridos, colesterol total (CT) y colesterol asociado a lipoproteínas de alta densidad (cHDL) en plasma fueron analizados usando el analizador Hitachi 911 (Roche Diagnostics, Indianapolis, Ind, USA). El colesterol asociado a lipoproteínas de baja densidad (cLDL) fue calculado usando la fórmula de Friedewald[22] ajustada para los niveles de triglicéridos plasmáticos.[23] La calidad de los ensayos bioquímicos fue controlada por el Servicio Andaluz de Salud.

Análisis genético

Mediante técnicas habituales se procedió a la obtención de ADN a partir de leucocitos de muestras de sangre periférica y a su almacenamiento a -70° C hasta su posterior análisis. Se procedió al genotipado para polimorfismos habituales de los genes *APOE* y *APOC3* mediante la técnica de Polymerase Chain Reaction (PCR) y posterior digestión específica (Hha I y SstI, respectivamente), seguida de electroforesis. Se determinaron las frecuencias alélicas para

cada una de las isoformas S1, S2 de *APOC3* y ε2, ε3 y ε4 de *APOE*. El análisis genético se realizó en 538 adolescentes, de los que 420 completaron satisfactoriamente el test de Course Navette.

Determinación del nivel de riesgo lipídico-metabólico

Para analizar si la capacidad aeróbica estaba relacionada con el riesgo lipídico-metabólico en adolescentes, calculamos un índice de riesgo lipídico-metabólico definido por los niveles plasmáticos de glucemia, triglicéridos, cLDL, cHDL, y IMC. Cada una de estas variables fue tipificada en función del género y la edad, obteniendo una media 0 y desviación típica 1 para cada una de las variables. Los valores tipificados de cHDL fueron multiplicados por -1, de modo que para cada una de las variables, a mayor valor tipificado, mayor riesgo lipídico-metabólico. Semejantes índices han sido previamente utilizados en poblaciones similares[24] y mejoran la potencia para detectar asociaciones.[25]

El nivel de riesgo lipídico-metabólico fue computado como la suma de las variables anteriores, tipificadas. Por tanto, y por definición, la media del índice de riesgo lipídico-metabólico fue 0. A mayor puntuación en el índice, mayor riesgo lipídico-metabólico (valores mayores de 0 indican un incrementado riesgo). A menor puntuación, menor riesgo (valores menores de 0 indican un menor riesgo lipídico-metabólico).

Determinación de los niveles requeridos de capacidad aeróbica para prevenir riesgo lipídico-metabólico

Las ecuaciones estimando el índice de riesgo lipídico-metabólico en función de la capacidad aeróbica tipificada fueron determinadas, para cada variante genética, mediante regresión lineal simple ajustada por género, edad y maduración sexual. Usando dichas ecuaciones, el índice de capacidad aeróbica tipificado requerido fue calculado para satisfacer las ecuaciones para un índice de riesgo lipídico-metabólico = 0. Posteriormente, dicho índice de capacidad aeróbica fue des-tipificado para cada género y edad, teniendo en cuenta la media y desviación típica de capacidad aeróbica en cada grupo de edad y género. La fórmula para des-tipificar y calcular la capacidad aeróbica requerida fue: capacidad aeróbica requerida (paliers) = media + (Z x SD), donde Z es el índice de capacidad aeróbica tipificada requerida tras resolver las ecuaciones, media es la media del test de Course Navette (expresado en paliers) para cada grupo de edad y género, y SD es la correspondiente desviación típica.

Análisis estadístico

Las diferencias inter-grupos según el género fueron analizadas mediante el test de Student (en muestras con distribución normal) o el test de Mann Whitney (en muestras con distribución no normal). El modelo lineal general fue utilizado para analizar la relación entre el nivel de capacidad aeróbica y el índice de riesgo lipídico-metabólico tanto en niños como en niñas, tras ajustar por edad y maduración sexual. Para calcular las ecuaciones predictoras del índice de riesgo lipídico-metabólico en función de la capacidad aeróbica tipificada, se utilizó el método de regresión lineal para cada variante genética, ajustada por género, edad y maduración sexual. Los tests fueron considerados significativos al nivel $p<0,05$.

4.1.3. Resultados

La capacidad aeróbica fue mayor en niños que en niñas (Figura 1). En el grupo estudiado, tanto en niños ($p<0,001$) como en niñas ($p=0,012$), la capacidad aeróbica se relacionó inversamente con el nivel de riesgo lipídico-metabólico utilizado, tras ajustar por edad y maduración sexual (Figura 2).

Con objeto de analizar las interacciones entre factores genéticos y capacidad aeróbica en adolescentes, determinamos las ecuaciones y funciones de regresión lineal de las relaciones entre capacidad aeróbica y riesgo lipídico-metabólico para diferentes variantes genéticas (Figura 3). Dicho análisis, que fue realizado en conjunto para niños y niñas fue ajustado por género, edad y maduración sexual. Los datos sugieren que los adolescentes portadores de los alelos S2 del gen *APOC3* y ε4 del gen *APOE* requieren mayores niveles de capacidad aeróbica para presentar un bajo riesgo lipídico-metabólico ($p<0,05$). Sin embargo, los portadores del alelo ε2 del gen *APOE* no requieren ningún nivel específico de capacidad aeróbica, ya que presentaron niveles lipídico-metabólico saludables independientemente de la capacidad aeróbica que presentaban (Figura 3).

Con objeto de calcular los niveles mínimos de capacidad aeróbica asociados a un bajo riesgo lipídico-metabólico, se usaron las ecuaciones predictoras de dicho riesgo en función de la capacidad aeróbica, para cada variante genética. Los valores mínimos se obtuvieron al resolver dichas ecuaciones para obtener un nivel de riesgo lipídico-metabólico = 0, y están expresados en la tabla 2. Como puede apreciarse, dichos niveles son mayores para los portadores de los alelos S2 de *APOC3* y ε4 de *APOE*. De los 538 adolescentes evaluados genéticamente, 175 (33%) eran portadores del alelo S2 de *APOC3* o del alelo ε4 de *APOE*. Ello sugiere que el 33% de la población analizada requería de mayores niveles de capacidad

aeróbica para presentar un bajo nivel de riesgo lipídico-metabólico, en comparación con los portadores de los genes no mutados.

4.1.4. Discusión

El presente estudio es pionero en analizar interacciones entre capacidad aeróbica y variantes genéticas en relación a riesgo metabólico-cardiovascular en adolescentes españoles. Además presentamos, en función de variables genéticas, los valores mínimos requeridos de capacidad aeróbica asociados a un bajo riesgo lipídico-metabólico. Dichos valores están expresados en paliers alcanzados en el sencillo y ampliamente usado (en colegios e institutos) test de Course Navette, por lo que los datos del presente estudio presentan una importante aplicación práctica para la prevención de riesgo metabólico-cardiovascular desde la etapa adolescente.

Nuestros datos son transversales y, por tanto, no se pueden derivar de ellos relaciones causales en cuanto al efecto de una elevada capacidad aeróbica sobre el riesgo metabólico-cardiovascular en adolescentes. No obstante, otros estudios transversales consolidan nuestros resultados, mostrando una relación inversa entre el nivel de capacidad aeróbica y diferentes factores de riesgo cardiovascular durante la infancia y adolescencia.[26-28] A ello hay que añadir los hallazgos de estudios prospectivos que han establecido relaciones causales entre capacidad aeróbica y riesgo cardiovascular[5,6,29] Todos estos datos, tomados en conjunto, sugieren que un incremento de la capacidad aeróbica *per se* podría inducir a una disminución de riesgo cardiovascular. Basándose en esta relación, nuestro grupo de investigación estableció, por vez primera, los valores mínimos de capacidad aeróbica asociados a un bajo riesgo lipídico-metabólico en adolescentes[12] (Mesa et al., datos no publicados). Sin embargo, en dichos estudios no fueron contempladas variables genéticas que sí se han tenido en cuenta en el presente estudio.

Los polimorfismos SstI del gen *APOC3* y la variante ε4 del gen *APOE* son conocidos factores de riesgo lipídico-cardiovascular.[30,31] Es por ello que el presente estudio indica que los portadores de dichas variantes genéticas deberían hacer más ejercicio y alcanzar un mayor grado de capacidad aeróbica, en comparación con los portadores de los genotipos no mutados (*APOC3* S1/S1 y *APOE* ε3/ε3). Nuestros datos también sugieren que incluso en individuos genéticamente predispuestos a presentar riesgo metabólico-cardiovascular, el ejercicio físico aeróbico (realizado en mayor cantidad que en individuos con los genotipos *APOE* y *APOC3* no mutados) podría atenuar dicho riesgo. Estudios realizados en población adulta sugieren lo mismo y apoyan nuestros datos.[32,33] Sin embargo, nuestro estudio

debería ser replicado por otros estudios transversales en adolescentes y futuros estudios de intervención realizados en adolescentes serían necesarios para clarificar los resultados obtenidos en el presente trabajo. De hecho, recientes estudios de intervención realizados en adultos han mostrado que los efectos saludables del ejercicio físico sobre el riesgo lipídico-cardiovascular son modulados por el genotipo del gen *APOE*.[34,35] En relación al genotipo del gen *APOC3*, no existen estudios de intervención – que conozcamos – que analicen las interacciones entre *APOC3* y ejercicio físico sobre factores de riesgo cardiovascular. Es preciso indicar que, dadas las múltiples interacciones que pueden existir entre otros genes[36] y que pueden enmascarar posibles asociaciones, futuros estudios deberían ir encaminados a evaluar posibles interacciones entre actividad física en adolescentes y otros genes en relación al riesgo metabólico-cardiovascular.

La proporción de adolescentes portadores de los alelos S2 del gen *APOC3* o ε4 en el gen *APOE* fue del 33%. De ello se deduce que aproximadamente uno de cada tres adolescentes españoles puede estar precisando de una educación física especial, ya que sus genotipos indican una mayor predisposición hacia el riesgo metabólico-cardiovascular, lo cual podría ser compensado con una mayor capacidad aeróbica.

Este estudio da un paso adelante en cuanto a prevención de enfermedades metabólicas y cardiovasculares, aportando y sugiriendo la individualización de programas de intervención en adolescentes basados en aspectos genéticos. Igualmente, el presente trabajo pone de relieve la importancia de la actividad física incluso en individuos genéticamente predispuestos a un mayor riesgo metabólico-cardiovascular, en los que programas especiales de actividad física podrían ser aplicados. Dicha prevención debería comenzar en los colegios e institutos, ya que se encuentran en una posición inmejorable para promover la realización de actividad física en niños y adolescentes.

4.1.5. Referencias

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Tabla 1. Características de los sujetos participantes en el estudio.

	Niños (n=1034)	**Niñas** (n=1056)	**P**
Edad (años)	15,4 +/- 1,3	15,4 +/- 1,3	NS
IMC (kg/m^2)	21,7 +/- 3.5	21,5 +/- 3,3	NS
Capacidad aeróbica (paliers)	6,9 +/- 2,7	4,0 +/- 1,9	**<0,001**
Análisis bioquímico	(n=235)	(n=235)	
Triglicéridos (mg/dl)	71,4 +/- 31,4	65,8 +/- 27,2	**0,041**
LDL colesterol (mg/dl)	92,0 +/- 24,0	98,8 +/- 22,9	**0,002**
HDL colesterol (mg/dl)	50,7 +/- 9,6	59,2 +/- 11,6	**<0,001**
Glucosa (mg/dl)	95,4 +/- 8,8	91,3 +/- 8,3	**<0,001**
Análisis genético	(n=273)	(n=265)	
APOE ε2 alelo (%)	9,3	8,7	NS
APOE ε3/ε3 (%)	70,9	72,7	NS
APOE ε4 alelo (%)	19,8	18,6	NS
APOC3 S1/S1 (%)	83,3	81,8	NS
APOC3 S2 alelo (%)	16,7	18,2	NS

Valores expresados como media +/- desviación típica. IMC, índice de masa corporal; NS, diferencias no significativas entre género.

Tabla 2. Capacidad aeróbica necesaria para prevenir riesgo metabólico-cardiovascular en adolescentes, en función del género, edad, y variantes genéticas.

	APOC3		***APOE***	
	S1 homocigotos (82,8% de los adolescentes)	S2 alelo (17,2% de los adolescentes)	ε3 homocigotos (72% de los adolescentes)	ε4 alelo (19,6% de los adolescentes)
Niños (edad)				
13-14	5,6	7,3	5,8	7,7
14-15	6,2	8,0	6,4	8,3
15-16	6,7	8,4	6,9	8,8
16-18	7,1	8,9	7,3	9,3
Niñas (edad)				
13-14	3,1	4,2	3,2	4,4
14-15	3,7	4,9	3,8	5,1
15-16	4,2	5,5	4,3	5,7
16-18	4,3	5,6	4,4	5,8

Los valores de capacidad aeróbica están expresados en paliers del test Course Navette.

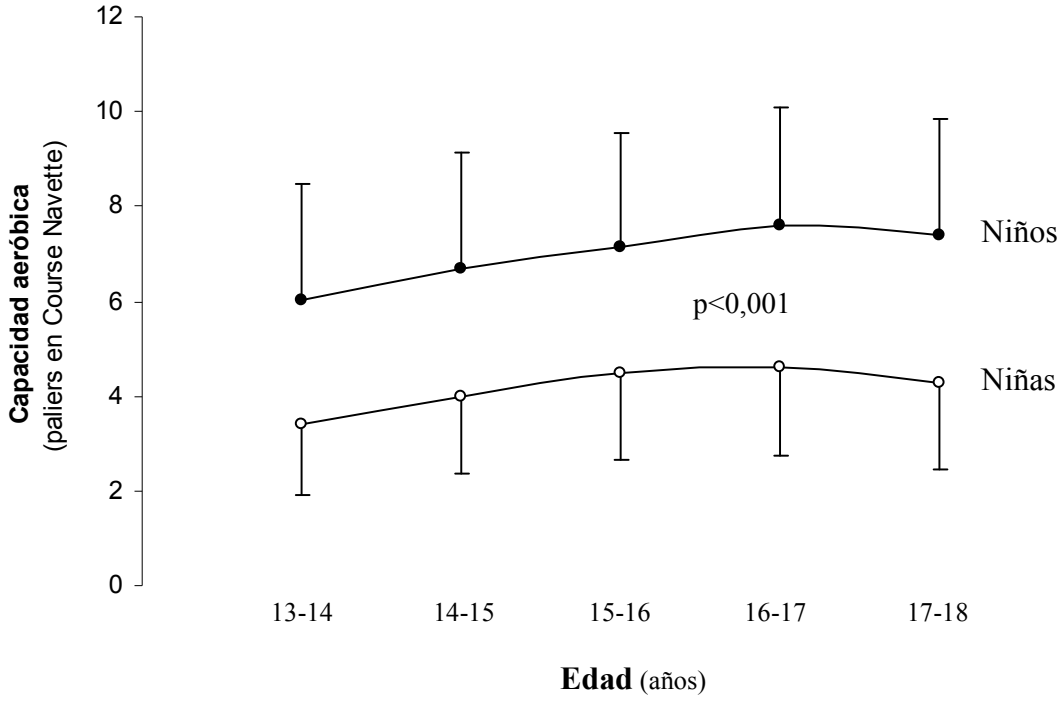

Figura 1. Capacidad aeróbica (expresada en paliers de Course de Navette) en niños (puntos negros, n=1034) y niñas (puntos blancos, n=1056). Los valores están expresados como media (puntos) y desviación típica (barras de error).

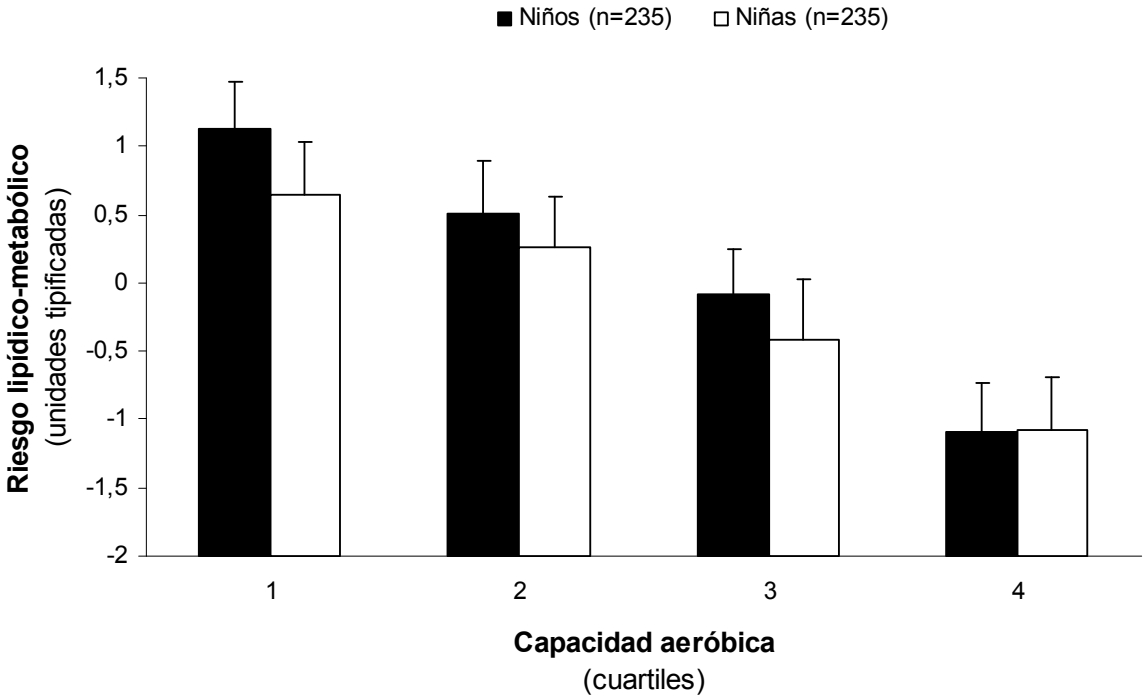

Figura 2. Relación entre capacidad aeróbica tipificada (cuarteles) y riesgo metabólico-cardiovascular, tras ajustar por edad y maduración sexual. Tanto en niños (P<0,001) como en niñas (P=0,012), existió una asociación significativa. Los valores están expresados en medias (barras) y error típico (barras de error).

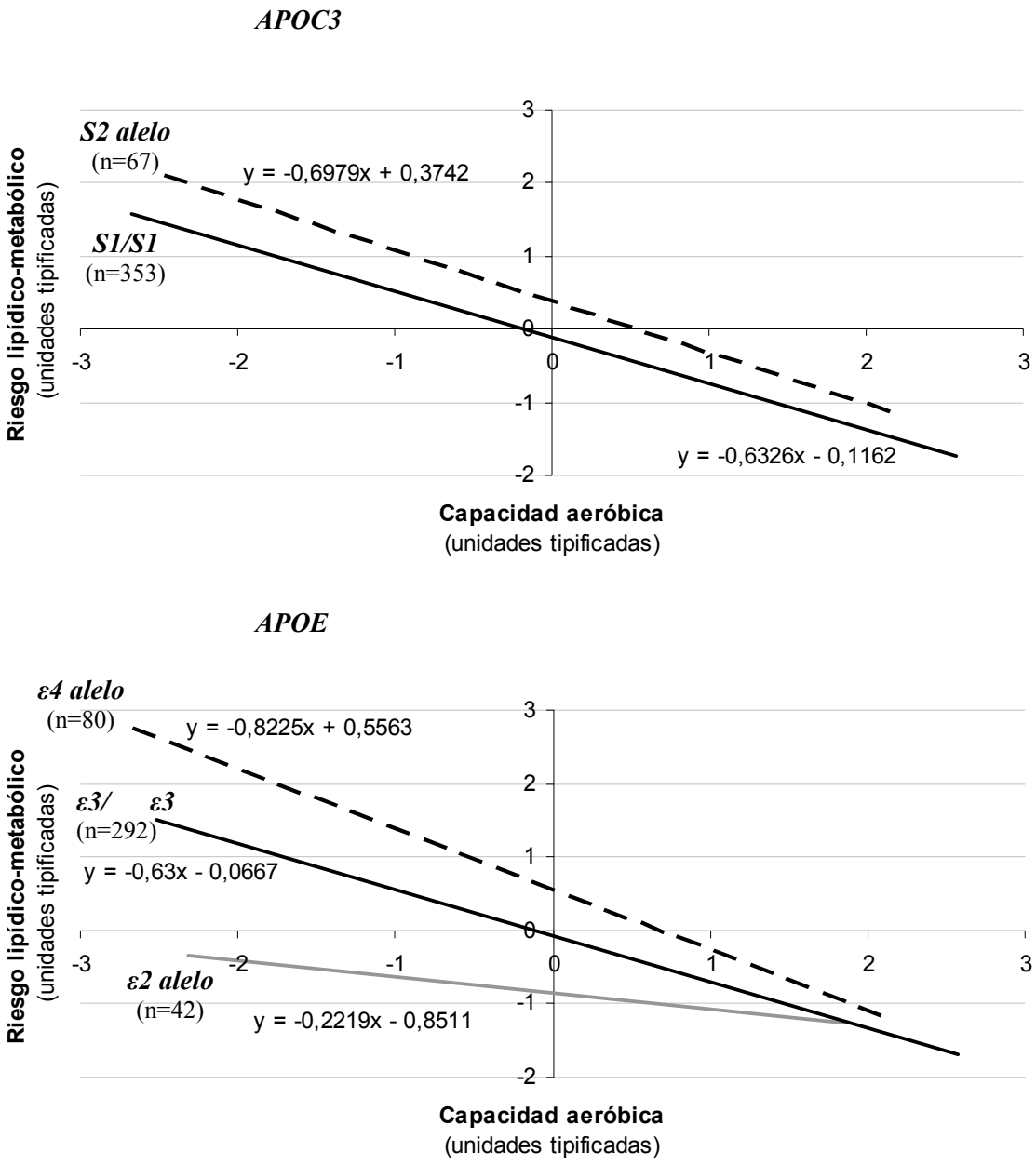

Figura 3. Ecuaciones predictoras del índice de riesgo metabólico-cardiovascular en función de la capacidad aeróbica tipificada, para cada variante genética. Todas las relaciones fueron significativas (P<0,05), salvo para el alelo ε2 del gen *APOE*.

4.2. Cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents

ABSTRACT

Aim: Firstly we tested whether a clustering of lipid-related cardiovascular risk factors in adolescents is related to mitogen-stimulated cytokine production from peripheral blood mononuclear cells (PBMCs). Secondly, we analysed any possible interaction of BMI, cardiorespiratory fitness, and common polymorphisms in *APOE* and *APOC3* genes on cytokine production from PBMCs.

Methods: Cardiorespiratory fitness, BMI, and a continuously distributed clustering of lipid-related atherogenesis risk factors (calculated from LDL and HDL cholesterol, triglycerides, glucose, lipoprotein(a) and apolipoprotein B-100) were measured in 470 adolescents (235 males, 235 females, 15.3+/-1.4 yr) from the cross-sectional multicentric AVENA study. In addition, *in vitro* cytokine production (IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10) was assessed in mitogen-stimulated PBMCs. *APOE* and *APOC3* genes were genotyped for common polymorphisms (ε2, ε3 and ε4 alleles and S1 and S2 alleles respectively).

Results: After adjustment for confounding factors, a high lipid-related cardiovascular risk score (4^{th} quartile), compared to lower risk level (2^{nd} quartile), was significantly associated with low levels of IL-6 (P=0.008) and IL-10 (P=0.002) production from mitogen-stimulated PBMCs. Following the same pattern, a trend of significance was found for TNF-α (P=0.081). No effect of weight status or cardiorespiratory fitness was observed on cytokine production in mitogen-stimulated PBMCs. IFN-γ, TNF-α, IL-2, IL-6 and IL-10 production from PBMCs was significantly lower in carriers of the S2 allele of *APOC3*. These differences remained significant even after adjustment for gender, age, sexual maturation, economic status, BMI, cardiorespiratory fitness, and lipid-related cardiovascular risk factors.

Conclusions: We show that an elevated clustering of lipid-related cardiovascular risk factors in adolescents is independently associated with an impaired mononuclear production of TNF-α, IL-6, and IL-10 in response to mitogens. We also show a general cytokine hyporesponsiveness in mitogen-stimulated PBMCs independently modulated by the *APOC3* SstI polymorphism in adolescents, suggesting a novel link between the immune system and cardiovascular risk in adolescents.

Key words: Immune system – Cardiovascular risk – Adolescents – APOC3

4.2.1. Introduction

Paediatric and adolescent obesity represents an uncontrolled and increasing worldwide epidemic (Ebbeling et al., 2002; Hedley et al., 2004; Moreno et al., 2005). Long-term health complications in overweight children and adolescents include diabetes, atherosclerosis and increased rates of cardiovascular diseases and mortality (Mossberg 1989; Must et al., 1992). In fact, subtle metabolic abnormalities predisposing to atherosclerosis and diabetes are already detected in overweight children and adolescents (Sinha et al., 2002; Weiss et al., 2004).

Evidence suggests that inflammatory processes and the immune response may play an important role in the early development of both cardiovascular disease and diabetes (Greaves & Channon, 2002; Kolb & Mandrup-Poulsen, 2005). Given the uncontrolled and increasing worldwide obesity epidemic and related metabolic disorders in children and adolescents (Ebbeling et al., 2002; Hedley et al., 2004; Moreno et al., 2005), studies analysing a possible role of the immune system on obesity-related metabolic disorders in adolescents are warranted, but quite scarce.

In this study we examine the associations between *in vitro* immune response (measured by cytokine production in mitogen-stimulated peripheral blood mononuclear cells (PBMCs)) and a clustering of lipid-related cardiovascular risk factors in adolescents. The advantage of examining the associations between immune response and lipid-related cardiovascular risk factors in adolescents is that there is no confounding by acute coronary disease, as the associations are established early in life.

Since weight status, cardiorespiratory fitness and common polymorphisms in *APOE* and *APOC3* genes may influence cardiovascular risk (Hallman et al., 1991; Talmud et al., 1997; Boreham et al., 2001; Wärnberg et al., 2004; Weiss et al., 2004), we also tested for possible interactions of these variables with *in vitro* cytokine production from mitogen-stimulated PBMCs in relation to lipid-related cardiovascular risk.

4.2.2. Methods

Study population

A total of 470 healthy Caucasian adolescents from five different geographic locations from Spain took part in the study. Complete clinical, genetic, anthropometric, and

cardiorespiratory fitness data were available for all participants. The subjects were all participants in the AVENA Study (González-Gross et al., 2003; Moreno et al., 2003), a population-based cross-sectional multicentric study of the aetiology and pathogenesis of obesity and related metabolic disorders. Parents and school supervisors were informed by a letter about the nature and purpose of the study and they gave their written informed consent. The study protocol was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki, and approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain).

Blood sampling and biochemical tests

Blood collection was carried out between 8:00 and 9:00 a.m., and after an 8-h overnight fast. The subjects were selected randomly (according to age, sex, geographical location, and economic status) and were instructed to abstain from alcoholic beverages for at least two weeks before sampling and to refrain from vigorous exercise during the 48 h preceding blood collection. Within one hour after collection, blood was centrifuged and aliquots of sera were sent refrigerated to a central laboratory (Clinical Biochemistry Service, Granada University Hospital) where all the clinical chemistry tests were performed within 24 hours after collection. Plasma glucose, triglycerides and high density lipoprotein (HDL) cholesterol were measured by enzymatic assay using a Hitachi 911 Analyzer (Roche Diagnostics, Indianapolis, Ind, USA). For the HDL cholesterol assay, precipitation was done using reagents provided by Boehringer (Ingelheim, Germany). LDL cholesterol was calculated with the Friedewald formula (Friedewald et al., 1972) adjusted for plasma triglycerides levels (Nakanishi et al., 2000). Apolipoprotein (apo) B-100 and lipoprotein(a) [Lp(a)] were measured using a immunonephelometric assay on Array 306 system (Beckman GMI, Inc., Albertville, Minnesota, USA). Quality control of the assays was assured by the Regional Health Authority, as is compulsory for all hospital clinical laboratories in Spain.

Genotyping

Genomic DNA was isolated form leukocytes of whole blood samples according to standard procedures (Miller 1988). The extracted DNA was stored at -70°C until analysed. *APOE* genotyping was performed by means of polymerase chain reaction (PCR), specific digestion with H*hal* (Promega), and electrophoresis in 12% acrylamida gel. Genotype characterization for the polymorphic S*stI* (position 371) in the 3' untranslated region of the *APOC3* gene was performed by PCR, specific digestion of the amplified products with the restriction enzyme SstI (Promega), and electrophoresis in 2% agarose gel (Hixson et al., 1991).

Cytokine production from peripheral blood mononuclear cells

Cytokine production was assessed in cultured mitogen-stimulated PBMCs. 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 PBMCs 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 ml/ml) and lipopolysaccharide (1.5 ml/ml), 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 prior to analysis. Cytokine 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.

Body composition assessment

For anthropometric measurements, subjects were barefoot and in their underwear. Weight was measured with a Seca scale (precision of 50 g), and height with incorporated stadiometer to the scale (precision of 1 mm). Harmonization and standardization of anthropometric measurements used to assess body composition within the AVENA multicenter study was strictly controlled and has been previously published elsewhere (Moreno et al., 2003, 2005). To establish the overweight (including obesity) and non-overweight categories, we used the gender- and age-adjusted cut-off points provided by Cole et al. (2000).

Cardiorespiratory fitness assessment

Cardiorespiratory fitness was measured by the progressive 20-m shuttle run test (Léger et al., 1982). This test was validated for use in children about 20 years ago (van Mechelen et al., 1986). Since then, it has been used in schools worldwide to assess aerobic physical fitness in children and adolescents. This test required subjects to run back and forth between two lines set 20 m apart. Running pace was determined by audio signals, emitted from a pre-recorded cassette tape, the initial velocity being 8.5 $km \cdot h^{-1}$, and increasing by 0.5 $km \cdot h^{-1}$ every minute (step). The tape used was calibrated over 1 min duration and the tape machine was checked for accuracy prior to each test. Subjects were instructed to run in a straight line, to pivot upon completing a shuttle and to pace themselves in accordance to the time intervals. The test was finished when the subject failed to reach the end lines concurrent with the audio signals on two consecutive occasions. Scoring was by steps completed (precision of 0.5 steps). A constant level of encouragement was given to

participants throughout the test. Subjects were instructed to abstain from strenuous exercise over 48 h preceding the test.

Lipid-related cardiovascular risk score

To investigate whether the clustering of lipid-related cardiovacular risk factors was related to body composition variables, we decided to compute a continuous risk score from the following measurements: triglycerides, HDL cholesterol, LDL cholesterol, Lp(a), apo B-100 and glucose. For each of these variables, a Z score was computed as the number of SD units from the sample mean after normalization of the variables, i.e., Z = ([value - mean]/SD). The HDL cholesterol Z score was multiplied by -1 to indicate higher cardiovascular risk with increasing value. The lipid-related atherogenesis risk score was the sum of the four Z scores. The mean of this continuously distributed lipid-related cardiovascular risk score is therefore zero by definition.

Statistical analysis

Inter-groups differences among gender were assessed by either the Student's t test (parametric variables) or Mann Whitney U test (non-parametric variables). Generalized linear model analyses adjusted for confounding factors were used to assess the associations between cytokine production from PBMCs and a lipid-related cardiovascular risk score. To test the interaction between anthropometric variables, cardiorespiratory fitness, and genetic polymorphisms on both cytokine production from PBMCs and the cardiovascular risk score, further linear modelling was undertaken. The level of significance for all analyses was set at $P<0.05$.

4.2.3. Results

A high lipid cardiovascular risk score was associated with low production of TNF-α, IL-6 and IL-10

By using general lineal model analysis adjusted for confounding factors, no significant associations were found between the lipid-related cardiovascular risk score and the production of IFN-γ, IL-2, or IL-4 from mitogen-stimulated PBMCs. However, a high lipid-related cardiovascular risk score (4^{th} quartile), compared to low levels (2^{nd} quartile), was significantly associated with low levels of IL-6 ($P=0.008$) and IL-10 ($P=0.002$) production from PBMCs (figure 1). In addition, and following the same pattern, a trend of significance

was observed for TNF-α (P=0.081). These associations were found after adjustment for gender, age, BMI, sexual maturation, and economic status.

No interaction with weight status or cardiorespiratory fitness

General lineal model analysis was used to assess any effect of weight status and cardiorespiratory fitness on TNF, IL-6 and IL-10 production from PBMCs in relation to lipid cardiovascular risk factors in adolescents. Overweight and non-overweight categories were defined following the cut-off pints provided by Cole et al. (2000). Cardiorespiratory fitness data were stratified above (fit) and below (unfit) the gender- and age-adjusted mean for cardiorespiratory fitness. After adjustment for gender, age, sexual maturation and economic status, no effect of weight status or cardiorespiratory fitness was observed on cytokine production from PBMCs (figure 2). In addition, after adjustment for the same confounders, no interaction of weight status or cardiorespiratory fitness on the relationship between cytokine production and lipid cardiovascular risk factors was observed (figure 2).

APOC3 SstI polymorphism modulates cytokine production from PBMCs

Since *APOE* and *APOC3* polymorphisms were reported to affect lipid cardiovascular risk factors (Hallman et al., 1991; Talmud 1997), we assessed the effect of common *APOE* and *APOC3* polymorphisms in relation to both cytokine production and lipid cardiovascular risk factors in adolescents. No interaction of *APOE* or *APOC3* polymorphisms on cytokine production from PBMCs in relation to lipid cardiovascular risk factors was found (figure 3). However, an acute decrease in TNF (P=0.005), IL-6 (P=0.048) and IL-10 (P=0.132) production from PBMCs was found in subjects carrying the S2 allele of *APOC3* (figure 2). In order to explore whether such a decrease was also extended to other cytokines, we analysed the effect of *APOC3* SstI polymorphism on the complete cytokine profile. Except for IL-4, cytokine production from mitogen-stimulated PBMCs was significantly lower in carriers of the S2 allele of *APOC3* (table 2). The differences remained significant even after adjustment for gender, age, sexual maturation, economic status, BMI, cardiorespiratory fitness, and lipid-related cardiovascular risk factors (table 2).

4.2.4. Discussion

Examining associations between immune response and lipid-related cardiovascular risk factors in adolescents avoids any confounding by acute coronary disease, as the associations are established early in life. We report in this study that an elevated clustering of lipid-related cardiovascular risk factors in adolescents is independently associated with

an impaired production of TNF-α, IL-6, and IL-10 in mitogen-stimulated PBMCs. We also show that this hyporesponsiveness is generalized to other cytokines in adolescents with the *APOC3* SstI polymorphism, independently of confounders such as body weight, cardiorespiratory fitness, and lipid-related cardiovascular risk factors, among others. Thus, our data suggest a novel link between the immune system and cardiovascular risk mediated, at least in part, by the *APOC3* SstI polymorphism.

These results are not surprising, as it is known that lipoprotein lipase (LPL) has differential effects on several inflammatory pathways, and that inflammation plays an important role in the pathogenesis of atherosclerosis. It is also well documented that LPL is able to modulate tumour necrosis factor-alpha (TNF-alpha)- and interferon-gamma (IFN-gamma)-mediated inflammatory cytokine signal transduction pathways in human endothelial cells. (Kota et al., 2005). Apolipoprotein C-III (apoC-III), a major component of TG-rich lipoproteins, is an inhibitor of LPL (McConathy et al., 1992). The *Sst*I polymorphism in the 3' untranslated region of the *APOC3* gene has been reported to be associated with TG levels in a number of studies (Ordovas et al., 1991; Hoffer et al., 1998), but not in others (Kee et al., 1999), as has been the case for *LPL* gene variations. Therefore, it appears that additional genetic and/or environmental factors have an impact on the potential associations of these polymorphisms depending on the ethnic-geographical origin of the studied population (Corella et al., 2002).

Elevated plasma levels of cytokines (especially TNF-α and IL-6) in patients with cardiovascular disease, as well as in other obesity-related conditions, have been reported (Lindmark et al., 2001; Lim et al., 2004). Plasma levels of TNF-α and IL-6 originate from different sources, for example adipose tissue (Carey et al., 2004; Lappas et al., 2005), and do not necessarily reflect the ability of the immune system to respond to mitogen or infection stimuli *in situ*. On the contrary, an inefficient production of cytokines from the mononuclear cells upon mitogen or infection stimuli apparently represents a significant defect in the response of the immune system, and is likely to contribute to aberrations in the course of inflammation in cardiovascular disease, diabetes, and other obesity-related conditions.

Our data indicate for first time a decrease in TNF-α, IL-6 and IL-10 production from mitogen-stimulated PBMCs in adolescents with a high lipid-related cardiovascular risk. These cytokines are among the first cytokines released when the host encounters foreign invaders. They play an important role in mediating innate immunity either alone or collaborating with other cytokines. As a result, conditioning the body by elevating these mediators of innate immunity prepares the body to combat diseases effectively. Thus, our data suggest a defect in the functioning of the immune system in adolescents with a high

lipid-related cardiovascular risk score. Our data are in agreement with other studies showing that type 1 and type 2 diabetes, which are often accompanied with accelerated atherosclerosis, are associated to altered cytokine production from PBMCs (Kretowski et al., 2000; Zykova et al., 2000; Zykova et al., 2004) and whole blood (Pickup et al., 2000). The same pattern has been observed in gastric cancer patients (Siedlar et al., 2005). The hyporesponsiveness we observed in some individuals might be the result of the interaction of some environmental factors, such as previous prolonged *in vivo* contact of subjects' PBMCs with chemical mitogens or their products/derivatives. This is biologically plausible as there is accumulating evidence indicating that infection may be linked to atherosclerotic disease (Kol & Libby, 1998; Vita & Loscalzo, 2002) and blood lipid disturbances (Gidding et al., 1998; Khovidhunkit et al., 2004) and regulation of endothelial LPL could play a central role.

Although the true role of infection as a risk factor for atherosclerosis is unclear, several known mechanisms may play at least a partial role in this process. One of the most likely mechanisms involves lipopolysaccharides (LPS), which has been implicated in atherogenesis and lipid disturbances (Levels et al., 2005). Future studies are needed to clarify whether the hyporesponsiveness we observed here is due to chronically high blood LPS levels.

Various studies have suggested a close association of the S2 allele of the *APOC3* gene with lipid disturbances (Dammerman et al., 1993; Dallinga et al., 1997; Liu et al., 2004), cardiovascular disease (Ferns et al., 1985; Chhabra et al., 2004), and impaired insulin sensitivity (Salas et al., 1998; Pérez-Jiménez et al., 2002). Interestingly, we demonstrate here a novel link in adolescents between the *APOC3* SstI polymorphism and PBMCs hyporesponsiveness to mitogen stimulation *in vitro*, independently, among other confounding factors, of lipid-related cardiovascular risk factors. Adjustment for this confounding factor is important as triglyceride-containing lipoprotein binds and inactivates LPS, thus reducing the stimulus for cytokine release (Miles, 1993). Thus, our data suggest that adolescents carrying the S2 allele of *APOC3* may be predisposed to a defect in the functioning of the immune system that is likely to contribute to cardiovascular risk. Nevertheless, caution must be exercised in this possibility and further studies are needed to confirm our results. If confirmed, future studies would address the mechanisms by which *APOC3* may modulate cytokine response from PBMCs.

4.2.5. References

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Table 1. Characteristics of the study population.

	Males (n=235)	**Females** (n=235)	**P**
Age (years)	15.2 +/- 1.4	15.2 +/- 1.4	0.839
BMI (kg/m^2)	22.1 +/- 4.0	21.6 +/- 3.5	0.135
Cardiorespiratory fitness (steps)	6.9 +/- 2.7	4.0 +/- 1.9	**<0.001**
Lipid profile			
Triglycerides (mg/dl)	71.4 +/- 31.4	65.8 +/- 27.2	**0.041**
LDL cholesterol (mg/dl)	92.0 +/- 24.0	98.8 +/- 22.9	**0.002**
HDL cholesterol (mg/dl)	50.7 +/- 9.6	59.2 +/- 11.6	**<0.001**
Lipoprotein(a) (mg/dl)	30.5 +/- 36.1	32.1 +/- 38.7	0.633
Apo B-100 (mg/dl)	66.5 +/- 14.4	70.4 +/- 13.7	**0.003**
Glucose (mg/dl)	95.4 +/- 8.8	91.3 +/- 8.3	**<0.001**
Cytokine profile			
ln IFN-γ (pg/ml)	9.4 +/- 1.3	9.2 +/- 1.2	0.095
ln TNF-α (pg/ml)	7.5 +/- 0.9	7.4 +/- 0.8	0.267
ln IL-2 (pg/ml)	5.1 +/- 1.0	5.1 +/- 1.0	0.812
ln IL-4 (pg/ml)	4.1 +/- 0.8	4.2 +/- 0.8	0.198
ln IL-6 (pg/ml)	10.2 +/- 0.9	10.3 +/- 0.9	0.214
ln IL-10 (pg/ml)	6.2 +/- 1.0	6.2 +/- 0.9	0.612
Genetic profile			
APOE ε2 carriers (%)	9.6	8.2	NS
APOE ε3/ε3 (%)	70.0	73.6	NS
APOE ε4 carriers (%)	20.4	18.2	NS
APOC3 S1/S1 (%)	83.3	81.8	NS
APOC3 S2 carriers (%)	16.7	18.2	NS

Data are presented as means +/- SDs. Inter-group differences among gender were assessed by either the Student's t test (parametric variables) or Mann Whitney U test (non-parametric variables).

Table 2. Cytokine production from PBMCs according to *APOC3* SstI polymorphism (S1, S2) in adolescents (n=470) after successive adjustment for confounding factors.

	Adjustment for gender, age, sexual maturation and economic status			**Additional adjustment for BMI and cardiorespiratory fitness**			**Additional adjustment for lipid-related cardiovascular risk score**		
	APOC3 SstI polymorphism			*APOC3* SstI polymorphism			*APOC3* SstI polymorphism		
	S1 homozygotes	S2 carriers	P	S1 homozygotes	S2 carriers	P	S1 homozygotes	S2 carriers	P
IFN-γ (pg/ml)	12100 (864)	7935 (1339)	**0.014**	11755 (1004)	7376 (1490)	**0.021**	11743 (1003)	7754 (1576)	**0.042**
TNF-α (pg/ml)	1878 (94)	1135 (130)	**<0.001**	1897 (113)	1159 (160)	**0.001**	1895 (115)	1167 (164)	**0.001**
IL-2 (pg/ml)	176 (10)	123 (18)	**0.014**	169 (12)	118 (20)	**0.038**	168 (13)	118 (21)	**0.042**
IL-4 (pg/ml)	61 (3)	57 (6)	NS	60 (3)	55 (4)	NS	60 (4)	54 (4)	NS
IL-6 (pg/ml)	29525 (1545)	20764 (2508)	**0.005**	29762 (1841)	20394 (2901)	**0.010**	29703 (1837)	20682 (2989)	**0.015**
IL-10 (pg/ml)	508 (29)	372 (49)	**0.022**	523 (36)	366 (58)	**0.028**	521 (35)	377 (60)	**0.047**

Data are presented as geometric means (SEMs). BMI is body mass index (kg/m^2); PBMCs, peripheral blood mononuclear cells.

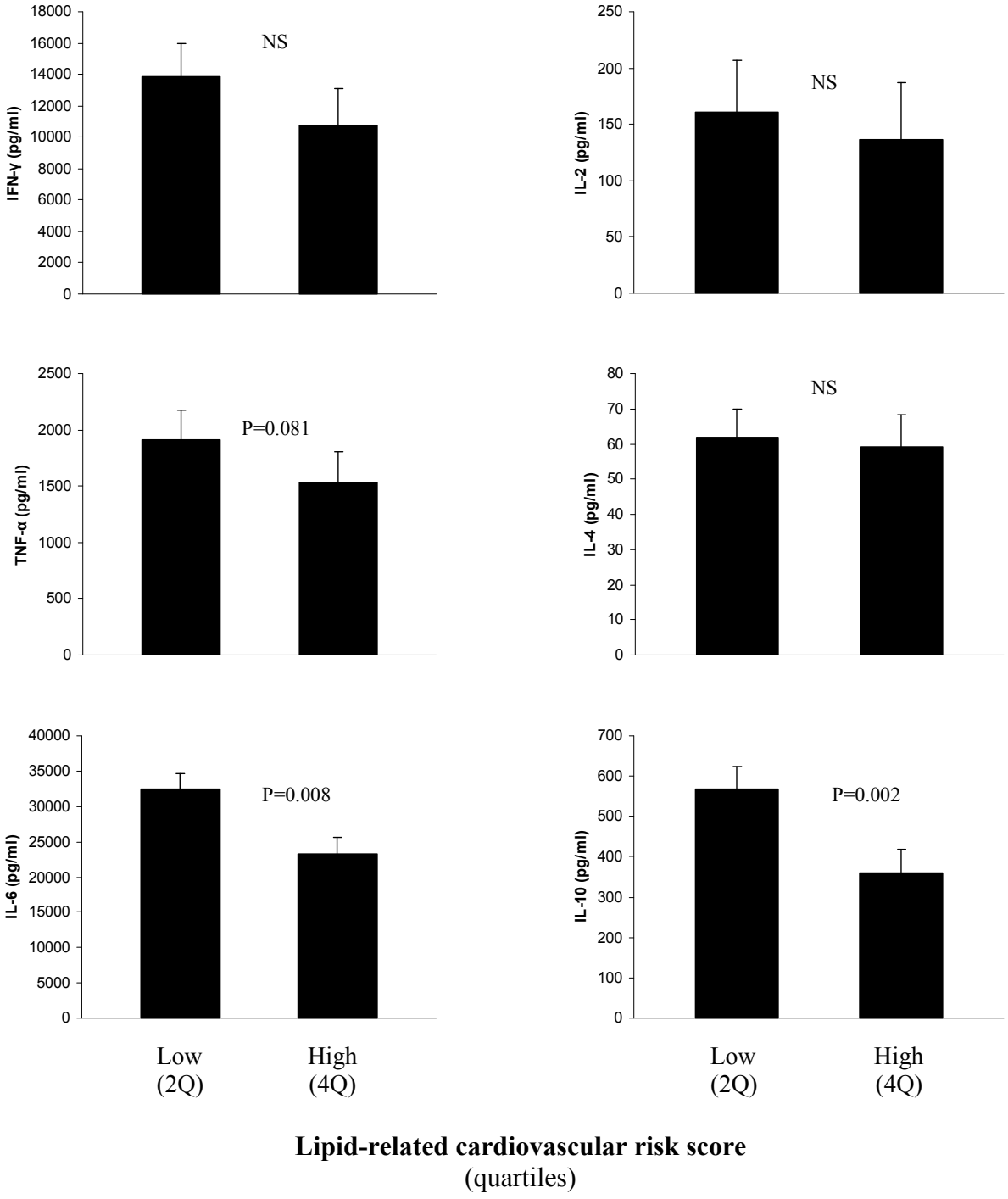

Figure 1. Associations between low (2nd quartile, n=120) and high (4th quartile, n=115) levels of a lipid-related cardiovascular risk score and cytokine production from PBMCs in adolescents. Data are presented as geometric means (bars) and SEMs (error bars) after adjustment for gender, age, BMI, sexual maturation, and economic status.

BMI is body mass index (kg/m^2); PBMCs, peripheral blood mononuclear cells; SEM, standard error of measurement.

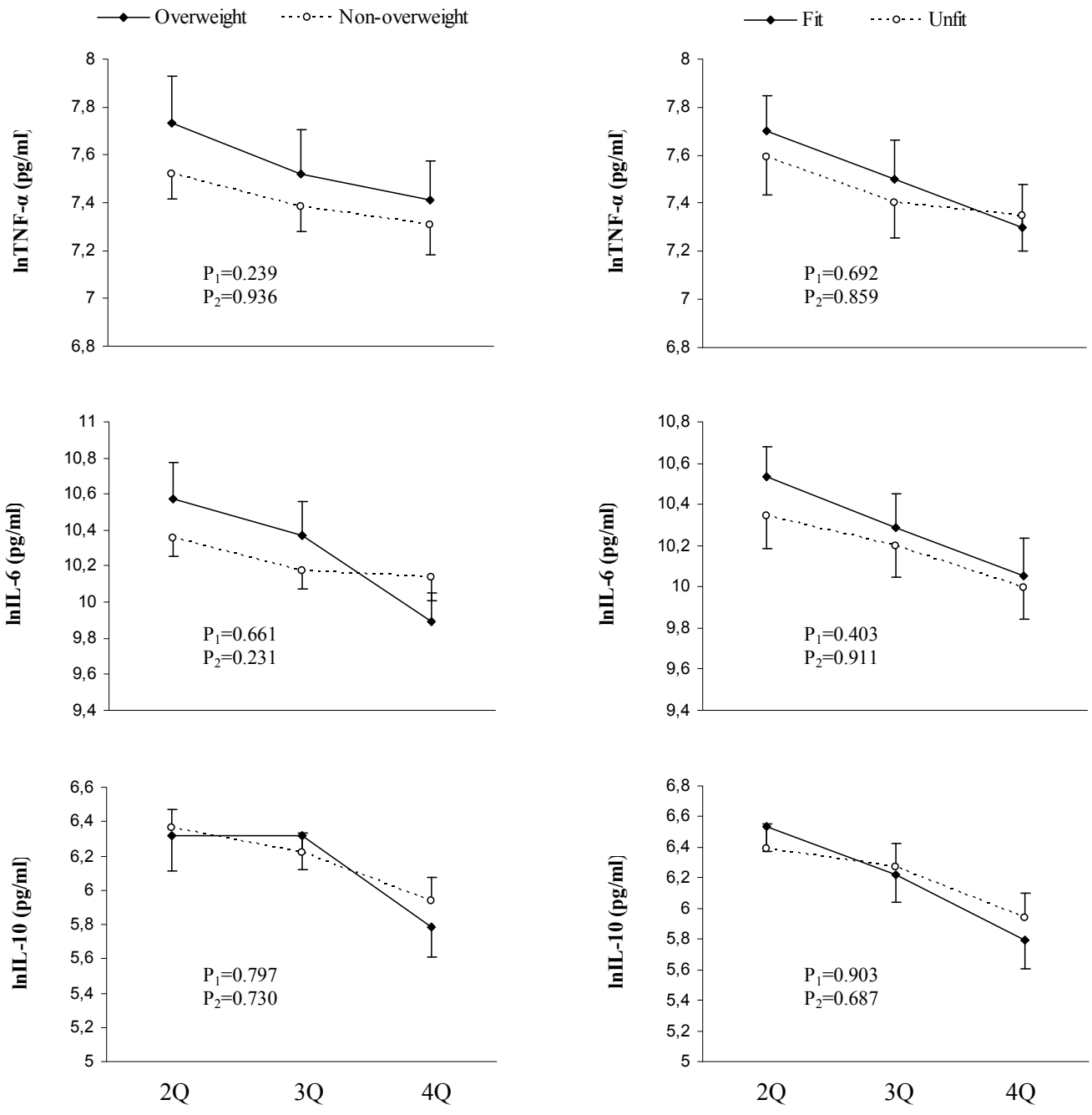

Figure 2. Effects of weigh status and cardiorespiratory fitness on the relationships between cytokine production from PBMC and a lipid-related cardiovascular risk score (quartiles) in adolescents (n=350), after adjustment for confounding factors. Overweight and non-overweight categories were defined following the cut-off pints provided by Cole et al. (2000). Cardiorespiratory fitness data were stratified above (fit) and below (unfit) the gender- and age-adjusted mean for cardiorespiratory fitness. P_1 refers to the effect of weight status or cardiorespiratory fitness on cytokine production from PBMC. P_2 refers to weight status or cardiorespiratory fitness interaction with lipid-related cardiovascular risk factors in relation to cytokine production from PBMC.

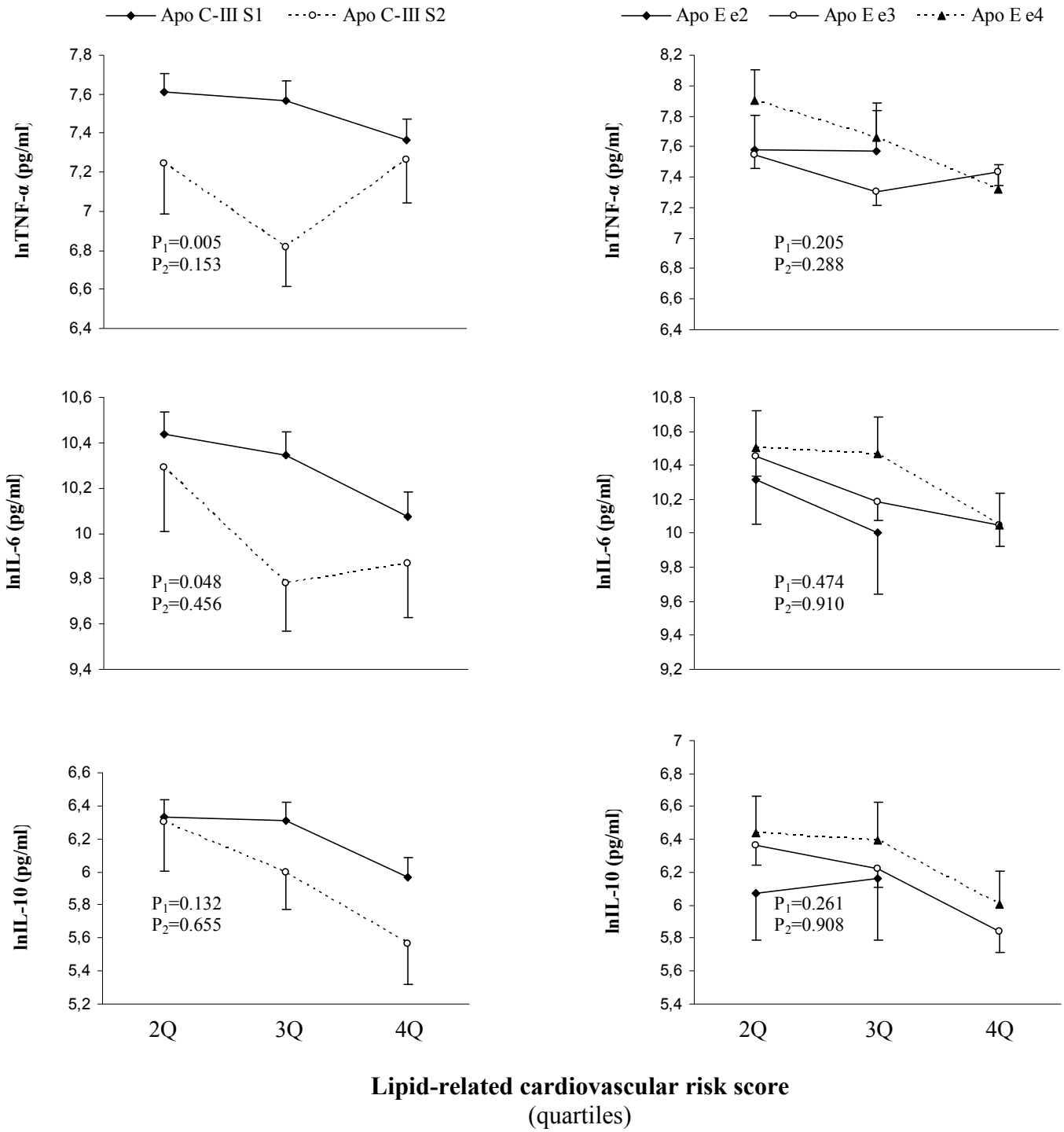

Figure 3. Effects of *APOC3* SstI and *APOE* polymorphisms on the relationships between cytokine production from PBMC and a lipid-related cardiovascular risk score (quartiles) in adolescents (n=350), after adjustment for confounding factors. P_1 refers to the effect of *APOC3* or *APOE* polymorphisms on cytokine production from PBMC. P_2 refers to the interaction of *APOC3* or *APOE* polymorphisms with lipid-related cardiovascular risk factors in relation to cytokine production from PBMC.

5.

General comments and conclusions

In this PhD Thesis 4 important outcomes have been raised:

1) We set minimal levels of aerobic physical fitness associated to a favourable lipid profile in male adolescents.

2) We demonstrate the usefulness of suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk in adolescents.

3) We provide genotype-dependent aerobic physical fitness levels associated to a favourable lipid-related metabolic profile in adolescents.

4) We suggest that cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents, regardless physical fitness or weight status.

These general results underscore the importance of physical fitness in the prevention of obesity-related metabolic disorders and their interrelation with common gene variants on lipid- and immune-related metabolic outcomes.

5.1. The importance of aerobic physical fitness

In this PhD Thesis we underscore the importance of aerobic physical fitness in relation to lipid-related metabolic risk in adolescents. The findings of this PhD Thesis are in concordance with a number of recent cross-sectional and prospective cohort studies elegantly summarized (Ruiz et al., 2006). These studies suggest that high aerobic physical fitness is associated with a healthier metabolic profile in children and adolescents and that high cardiorespiratory fitness during these stages of life seems to provide more health protection in adulthood.

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NOTIFICATION

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The importance of cardiorespiratory fitness for healthy metabolic traits in children and adolescents: the AVENA Study

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The protective effect of physical activity on total mortality, life expectancy, cardiovascular disease and diabetes has been widely reported in people of all ages (Strong et al. 2005; Jonker et al. 2006; Franco et al. 2005). Recent estimates suggest that obesity and physical inactivity are responsible for 400,000 deaths annually in the United States; thus, it is close to overtaking tobacco as the leading cause of preventable death (Mokdad et al. 2004).

Cardiorespiratory fitness is a direct marker of physiological status and recent data suggest that fitness is one of the strongest predictors of health outcomes (Myers et al. 2002; Mora et al. 2003). In fact, cardiorespiratory fitness is a direct measure of physiological performance and of the ability to adapt to physical stress. Physical activity and cardiorespiratory fitness are closely related in that fitness is partially determined by physical activity patterns over recent weeks or months. Cardiorespiratory fitness is also determined by constitutional factors. It has been suggested that ~40% of variation in cardiorespiratory fitness is attributable to genetic factors (Bouchard 1986).

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Low cardiorespiratory fitness may threaten, together with the present obesity epidemic, to shorten life expectancy over the next few decades (Olshansky et al. 2005). Therefore, cardiorespiratory fitness has been suggested to be included in the European Health Monitoring System for the adult population (Sjöström et al. 2005). Ruiz et al. (2006) suggested that cardiorespiratory fitness should also be included in the health monitoring systems from the early stages of life. The results from the Swedish and Estonian part of the European Youth Heart Study (EYHS) revealed negative associations between cardiorespiratory fitness and features of metabolic syndrome in girls and boys aged 9–10 years (Ruiz et al. 2006). The findings are in concordance with a number of recent cross-sectional and prospective cohort studies elegantly summarized in the manuscript. They suggest that high cardiorespiratory fitness is associated with a healthier metabolic profile in children and adolescents and that high cardiorespiratory fitness during these stages of life seems to provide more health protection in adulthood.

Similar results have been observed in a representative sample of Spanish adolescents participating in the "Alimentación y Valoración del Estado Nutricional de los Adolescentes" (AVENA) study (Gonzalez-Gross et al. 2003a; Moreno et al. 2003). The AVENA study is a population-based cross-sectional survey conducted in five different geographic areas of Spain, addressing genetic and environmental factors in relation to metabolic traits during adolescence. Some interesting data regarding cardiorespiratory fitness and cardiovascular risk factors are being obtained from this study.

First findings from the AVENA pilot study revealed a negative association between body fat derived from the sum of four skinfolds and cardiorespiratory fitness in both boys and girls aged 13–18.5 years (Gonzalez-Gross et al. 2003b). After analyzing the total sample of participants (n=2859; 1357 boys, 1502 girls) we found that moderate to high levels of cardiorespiratory fitness were associated with lower abdominal adiposity (as measured by waist circumference) in both genders (Ortega et al. unpublished data). This association remained after adjustment for age, pubertal

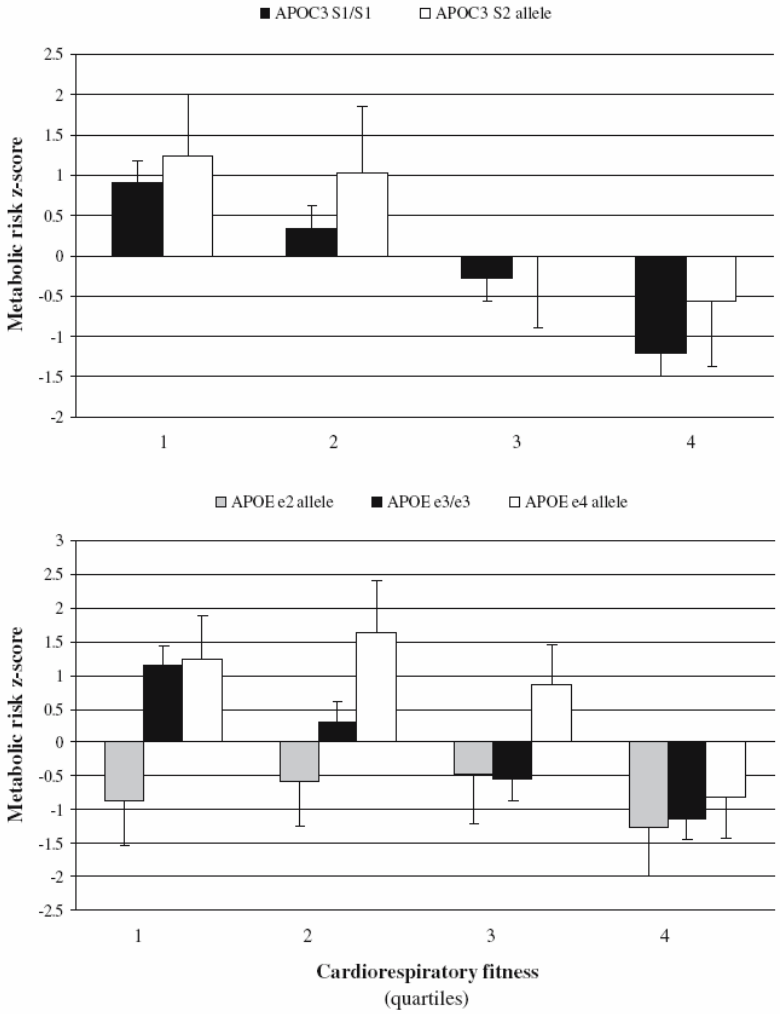

Fig. 1 General linear model showing genotype-dependent associations between cardiorespiratory fitness quartiles and metabolic risk score (computed as a typified z-score including values of triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, glucose and body mass index). Data are shown as mean and standard error of mean. All the associations were significant except for the apolipoprotein (APO) E e2 allele

maturation status and confounding factors (height, socioeconomic status, leisure time physical activity and active commuting to school). Associations between increased cardiorespiratory fitness and a favourable metabolic profile in both overweight and non-overweight adolescents have also been found, and the main outcome was that cardiorespiratory fitness was an indicator of a favourable metabolic profile in male adolescents (Mesa et al. 2003).

In addition, we have fixed the minimal required values of cardiorespiratory fitness associated to a healthy lipid profile in adolescents (Mesa et al. in press). We fixed these values using the widely used Course Navette (shuttle run test). Of note, 50% of the male population presented cardiorespiratory fitness values below the standards we fixed. Moreover, we have detailed the levels of cardiorespiratory fitness according to common apolipoprotein genetic variants (Mesa et al. unpublished data). This study emphasizes the importance of setting the required cardiorespiratory fitness levels according to genetic variants (Fig. 1). Future studies should address this issue, as the phenotype is the result of the interaction of both genetic and environmental factors.

On the basis of cardiorespiratory fitness (Ortega et al. 2005), approximately 20% of Spanish adolescents did not achieve the health-related threshold values of cardiorespiratory fitness (The Cooper Institute for Aerobics Research 1999). These data together with those showing increased body fat (Moreno et al. 2006) and impaired lipid profile (Ruiz et al. in press) indicate that special attention should be paid to this crucial period of life.

In summary, cardiorespiratory fitness is an important health marker not only in adults but also in children and adolescents. Therefore, it would be worthy to include cardiorespiratory fitness assessment in a pan-European health monitoring system from the early stages of life.

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5.2. Preventing overweight and obesity

We have identified in this PhD Thesis suprailiac skinfold thickness (in males) and waist-to-height ratio (in females) were the best simple anthropometric estimators of a clustering of lipid-related metabolic traits in a population of adolescents from Spain, mainly in non-overweight individuals and regardless their cardiorespiratory status. These simple measurements should be used for early identification and characterization of metabolic complications in adolescents. In addition, we propose several actions to prevent the childhood obesity epidemic.

What should mothers do?

It has been suggested that prenatal overnutrition might affect lifelong risk of obesity (Whitaker & Dietz, 1998). Besides, intrauterine exposure to inhaled smoke products rather than lifestyle factors associated with maternal smoking is an independent risk factor for subsequent childhood obesity (von Kries et al., 2002). Therefore, prospective mothers should adopt healthy nutritional habits and do not smoke (neither actively nor passively) since before conception. Children who were bottle fed seem to be more at risk of obesity later in childhood than those who were breast fed, after adjustment for socioeconomic status, birthweight and sex (Armstrong & Reilly, 2002). Thus, babies should be breast fed.

Replacing television viewing for physical activity

Reducing television, videotape, and video game use may be a promising approach to prevent childhood obesity (Robinson, 1999). Television viewing may promote weight gain not only by displacing physical activity, but also by increasing energy intake. Children seem to passively consume excessive amounts of energy-dense foods while watching television. Additionally, television viewing including 30-second food commercials influence preschool children's food preferences (Borzekowski & Robinson, 2001). Unfortunately, most food commercials aimed at children are for fast food, soft drinks, sweets, and sugar-sweetened breakfast cereal. Thus, parents should limit their preschooler's exposure to television, and replace it for physical activity, which has greatly diminished in technologically advanced, car- driving, telecommuting nations.

What physical activity?

In a recent meta-analysis (Le Mura & Maziekas, 2002), when the data were partitioned by exercise intensity, duration, mode, and intervention program, was found that a low exercise intensity (60-65% maximal oxygen uptake; e.g., swimming walking, running or cycling

moderately), a long exercise duration (>30 min), the combination of aerobic plus high-repetition (8–12 repetitions) resistance exercise, and an intervention of exercise plus behaviour modification resulted in the greatest decreases in percent body fat in children.

Attained the previous measures, the childhood obesity epidemic would be on the rack. In order to checkmate it, other comprehensive actions by many sectors of society are needed:

Educators' and health officials' actions

1) Children spend great time at schools, so educators and teachers should instil healthy habits in them. A good starting point could be to remove junk food commercials on school television, soft-drinks machines, and educational materials sponsorships of soft-drink and snack-food industries. Of course, governments should recompense schools for these actions. 2) Educators and health officials should advise parents to limit their children's television and video game utilization, and replace it for physical activity. Furthermore, they should train parents in healthy habits of nutrition and exercise. 3) Health officials should support several national or international no-TV or no-car/bus weeks per year. Additionally, they should support walking, jogging, swimming or cycling weeks.

Urban development actions

Urban planners and architects should build communities in which walking and bicycling are safe and convenient. For this, suitable sidewalks or bike paths would be needed. Besides, cities, towns, and buildings could be designed to help people lead healthier lives (e.g., making stairs and stairwells more accessible and attractive to use). Local governments should build attractive facilities to entice young people to walk and jog more, play tennis and basketball, and lift weights.

Government actions

1) Governments should counterbalance the food industry's marketing effort. Junk food should not be sold in schools, and fast food commercials should clearly state their unhealthy properties. Chain restaurants and manufactures should list caloric contents on menus and products, as well as put recognisable good-food symbols on products that meet specific criteria. 2) Governments should launch mass-media campaigns to promote better diets and more physical exercise for children, healthy habits for prospective mothers, and basic knowledge on childhood obesity for parents. 3) It would be desirable to increase the number of hours per week of physical education in schools and fund them to involve children in routine physical activity.

In order to develop effective comprehensive public policy response to the childhood obesity epidemic, we must expand its psychological, physical and economic sequelae as well as its treatment to society and politicians; otherwise, we and our children will live in an overweight population, experiencing such sequelae.

5.3. Genes and physical fitness on lipid-related metabolic traits

We found genotype-dependent associations between apoprotein genes (*APOC3* and *APOE*) and physical fitness in relation to metabolic traits. Our data are biologically plausible, as one intervention study (Hagberg et al., 1999) has reported a greater increase in HDL cholesterol levels in *APOE ε2* carriers compared to carriers of the isoform *ε3* and *ε4* after a 9-month endurance exercise period. In addition, both cross-sectional and longitudinal studies generally indicate that APO E2 and E3 individuals improve plasma lipoprotein-lipid profiles more with exercise training than APO E4 individuals (Hagberg et al., 2000).

Regarding possible interrelations of the immune system in these associations, we found that an elevated clustering of lipid-related cardiovascular risk factors in adolescents is independently associated with an impaired mononuclear production of TNF-α, IL-6, and IL-10 in response to mitogens. We also show a general cytokine hyporesponsiveness in mitogen-stimulated PBMCs independently modulated by the *APOC3* SstI polymorphism in adolescents. These associations were independent of weight or physical fitness status in adolescents. This suggests that adolescents carrying the S2 allele of *APOC3* may be predisposed to a defect in the functioning of the immune system that is likely to contribute to cardiovascular risk. These results, taken with caution, suggest a novel link between the immune system and cardiovascular risk in adolescents.

Some methodological issues have to be taken into account when researching gene and environment interactions in relation to metabolic traits. Observational studies of gene-environment interactions are informative about the manner in which genetic variation modifies the association between free-living lifestyle factors and disease. Thus, evidence from observational studies is likely to be most important for understanding the level of risk within a population that can be attributed to gene-environment interactions. However, to determine causality and to ascertain whether treatment can be optimised by targeting specific genetic subgroups requires randomised clinical trials (RCTs). To date, no RCT designed specifically for the purpose of testing gene-environment interactions has been reported.

5.4. Sumary box

What is already known on this topic	What this PhD Thesis adds
There are studies showing associations between physical fitness and lipid variables in adolescents. However, to our knowledge there are no studies providing minimal criterion standards of aerobic fitness in adolescents associated to healthy lipid-related metabolic variables.	We set minimal levels of aerobic physical fitness associated to a favourable lipid profile in male adolescents.
BMI, waist circumference, and waist-to-height ratio have been proposed as simple anthropometric measures related to metabolic and cardiovascular risk factors in children and adolescents. However, there are some studies casting some doubts about the value of these measurements to accurately explain lipid-related metabolic variables.	We demonstrate the usefulness of suprailiac skinfold thickness in males and waist-to-height ratio in females as simple anthropometric measurements associated to an overall lipid-related metabolic risk in adolescents.
Common apolipoprotein variants of *APOC3* and *APOE* genes influence lipid-related metabolic traits. However, to our knowledge, there are no studies analysing possible interactions between these genes and physical fitness in relation to lipid-related metabolic traits in adolescents.	We provide genotype-dependent aerobic physical fitness levels associated to a favourable lipid-related metabolic profile in adolescents.
Several studies in adolescents have assessed complex relationships between fitness, fatness and inflammation. However, to our knowledge there are no studies in this population analysing possible interactions with common apolipoprotein gene variants.	We suggest that cytokine production in mitogen-stimulated peripheral blood mononuclear cells is modulated by *APOC3* SstI polymorphism and is inversely related to lipid cardiovascular risk in adolescents, regardless physical fitness or weight status.

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(Annex)

Potential novel physiological mechanisms underlying exercise-induced beneficial metabolic effects

The importance of physical fitness and, hence, physical exercise, in relation to healthy metabolic outcomes in adolescents was emphasized in the previous sections. In the annex of this PhD Thesis we analyse possible and novel physiological mechanisms able to explain, at least in part, the exercise-induced beneficial metabolic effects. We focuss on two molecules widely studied over the last years: heat shock proteins (HSPs) and interleukin-6 (IL-6).

What is already known on this topic

Physical exercise can positively influence classical cardiovascular risk factors such as diabetes, hypertension, obesity, dyslipidemias, and endothelial dysfunction.

The physiological and molecular mechanisms are not fully understood, although increased lipolysis, GLUT4 translocation, and antioxidant capacity have been reported with physical exercise.

What this PhD Thesis adds

We provide the possibility that exercise may induce beneficial metabolic effects (mainly in glucose homeostasis) through acutely and systemically increased heat shock proteins and interleukin-6.

A.1. Introduction

Heat shock proteins (HSPs) or stress proteins are a highly evolutionary conserved family of polypeptides that are cytoprotective, protecting proteins, lipids, and nucleic acids from damage by reducing oxidation, preventing apoptosis, suppressing proinflammatory cytokines, repairing ion channels, and aiding in protein folding (Benjamin & McMillan, 1998). HSPs are low in individuals with type 2 diabetes, moderately low in the nondiabetic identical twin with a diabetic co-twin, and low in individuals with type 1 diabetes (Kurucz et al., 2002; Bruce et al., 2003). Furthermore, in a study comparing 5,600 genes of nondiabetic subjects with those of insulin-resistant diabetic subjects, HSP70 was 1 of only 17 genes that were markedly lower in individuals with diabetes (Patti et al., 2001). Importantly, a drug designed to increase HSP expression, bimoclomol, improves diabetic retinopathy, neuropathy, nephropathy, wound healing, cardiac ischemia, and insulin resistance in laboratory diabetic animal models (Vigh et al., 1997; Kurthy et al., 2002). Finally, heat therapy, via hot tub immersion, improves diabetic glycemic control and symptomatic diabetic neuropathy in patients with type 2 diabetes (Hooper, 1997). These data suggest that circulating HSPs may exert beneficial metabolic effects, mainly in glucose homeostasis.

In the annex of this PhD Thesis we firstly tested whether physical exercise increases plasma HSP levels, and if so, whether glucose ingestion influences the HSP response.

It is well know that physical exercise increases plasma plasma and muscle interleukin-6 (IL-6) levels (Ostrowski et al., 1998; Febbraio & Pedersen, 2002). However, the biological role of this increase is unknown. In the annex of this PhD Thesis we secondly tested *in vivo* the effects of IL-6 on glucose metabolism and insulin signalling pathways.

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This section comprises two studies analysing the effect of physical exercise on circulating heat shock proteins and testing a possible metabolic role of interleukin-6:

Glucose ingestion attenuates the exercise-induced increase in circulating HSP72 and HSP60 in humans

FEBBRAIO MA, **MESA JL**, CHUNG J, STEENSBERG A, KELLER C, NIELSEN HB, KRUSTRUP P, OTT P, SECHER NH, PEDERSEN BK. Published in *Cell Stress & Chaperones* 2004; 9: 390-396.

Prolonged interleukin-6 treatment improves glucose tolerance *in vivo* and does not result in activation of the NFκβ signalling pathway

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Glucose ingestion attenuates the exercise-induced increase in circulating heat shock protein 72 and heat shock protein 60 in humans

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Abstract Heat shock protein (Hsp) 72 is a cytosolic stress protein that is highly inducible by several factors including exercise. Hsp60 is primarily mitochondrial in cellular location, plays a key role in the intracellular protein translocation and cytoprotection, is increased in skeletal muscle by exercise, and is found in the peripheral circulation of healthy humans. Glucose deprivation increases Hsp72 in cultured cells, whereas reduced glycogen availability elevates Hsp72 in contracting human skeletal muscle. To determine whether maintained blood glucose during exercise attenuates the exercise-induced increase in intramuscular and circulating Hsp72 and Hsp60, 6 males performed 120 minutes of semirecumbent cycling at ~65% maximal oxygen uptake on 2 occasions while ingesting either a 6.4% glucose (GLU) or sweet placebo (CON) beverage throughout exercise. Muscle biopsies, obtained before and immediately after exercise, were analyzed for Hsp72 and Hsp60 protein expression. Blood samples were simultaneously obtained from a brachial artery, a femoral vein, and the hepatic vein before and during exercise for the analysis of serum Hsp72 and Hsp60. Leg and hepatosplanchnic blood flow were measured to determine Hsp72-Hsp60 flux across these tissue beds. Neither exercise nor glucose ingestion affected the Hsp72 or Hsp60 protein expression in, or their release from, contracting skeletal muscle. Arterial serum Hsp72 increased ($P < 0.05$) throughout exercise in both trials but was attenuated ($P < 0.05$) in GLU. This may have been in part because of the increased ($P < 0.05$) hepatosplanchnic Hsp72 release in CON, being totally abolished ($P < 0.05$) in GLU. Serum Hsp60 increased ($P < 0.05$) after 60 minutes of exercise in CON before returning to resting levels at 120 minutes. In contrast, no exercise-induced increase in serum Hsp60 was observed in GLU. We detected neither hepatosplanchnic nor contracting limb Hsp60 release in either trial. In conclusion, maintaining glucose availability during exercise attenuates the circulating Hsp response in healthy humans.

INTRODUCTION

Heat shock proteins (Hsps), highly conserved proteins found in all prokaryotes and eukaryotes, are molecular chaperones of naïve, aberrantly folded, or mutated proteins, as well as are essential to restore normal function and provide protection from disrupted cell homeostasis (Hartl 1996). The most abundant and widely studied families are the 60-kDa (Hsp60) and the 70-kDa (Hsp70) families (Bukau and Horwich 1998). Hsp60 is primarily mitochondrial in cellular location, playing a key role in the intracellular protein translocation and cytoprotection (Sigler et al 1998). Hsp70 is a cytosolic stress protein whose inducible form is Hsp72.

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Exercise results in an increase in serum concentration of Hsp72 (Walsh et al 2001; Febbraio et al 2002a) in part because of a release by the hepatosplanchnic viscera (Febbraio et al 2002a). During exercise, the rise in hepatic glucose production is suppressed by glucose ingestion because the demand for glucose is met by the ingested carbohydrate (Jeukendrup et al 1999b). Hence, the metabolic stress placed on the liver may be reduced under such circumstances, ultimately resulting in a decreased hepatosplanchnic Hsp72 release. Hsp60 has been found in the circulation of healthy individuals (Pockley et al 1999; Lewthwaite et al 2002), and the concentration of Hsp60 within the serum is associated with cardiovascular disease (Pockley et al 2000), high-density lipoprotein cholesterol, and tumor necrosis factor-α (Lewthwaite et al 2002). However, to our knowledge, no studies have examined the effect of physical exercise on the circulating Hsp60 response, the potential sources of circulating Hsp60 or whether glucose ingestion blunts this Hsp60 response. Therefore, the primary aim of the present study was to determine whether glucose ingestion would decrease the exercise-induced increase in serum Hsp60 and Hsp72 levels, and whether a reduction in hepatosplanchnic Hsp release may be responsible, in part, for any attenuation in the Hsp response.

Physical exercise may induce an increase in both Hsp72 and Hsp60 protein expression in a variety of tissues and mammalian species (Khassaf et al 2001; Kregel 2002). Interestingly, increased synthesis of glucose-regulated proteins (members of the Hsp family of proteins) occurs in Chinese hamster ovary cells deprived of glucose (Sciandra and Subjeck 1983). In addition, reduced glycogen availability is associated with elevated Hsp72 messenger ribonucleic acid (mRNA) and protein levels in contracting human skeletal muscle (Febbraio et al 2002c). It has been previously shown that 2 hours of bicycle exercise can deplete muscle glycogen (Febbraio et al 2000b), and because skeletal muscle glucose uptake and oxidation is increased by glucose ingestion (Febbraio et al 2000b) potentially sparing muscle glycogen use, another aim was to test the hypothesis that glucose ingestion would be capable of attenuating the exercise-induced increase in contracting muscle Hsp expression by decreasing the reliance on intramuscular glycogen.

MATERIALS AND METHODS

Subjects

Six healthy, active men (23.7 ± 6.3 years; 180 ± 5 cm; 74.9 ± 8.8 kg; maximal oxygen uptake [VO_{2max}] = 4.06 ± 0.10 L/min; mean ± SD) participated in the study, which was approved by the Ethical Committee of the Copenhagen and Frederiksberg Communities, Denmark, and performed according to the Declaration of Helsinki. Subjects were informed about the possible risks and discomfort involved before their written consent was obtained.

Preliminary testing

After the medical screening, each subject underwent a VO_{2max} test on a semirecumbent cycle ergometer. Semirecumbent cycling was chosen to allow for the determination of leg blood flow (LBF) using the thermodilution technique during the experimental trials. From this test, a workload was calculated, which would elicit ~65% of each individual's VO_{2max}. At least 3 days after the VO_{2max} test and 48 hours before the experimental trials, subjects reported to the laboratory and completed 45 minutes of upright cycling exercise at a workload corresponding to 65% of maximal heart rate. Thereafter, the subjects were provided with food packages, which they consumed for the next 2 days (15.6 MJ/day, ~70% CHO, 15% protein, 15% fat). During this period, the subjects were asked to adhere to the diet and to refrain from strenuous exercise and intake of alcohol, tobacco, and caffeine. This protocol was adopted to minimize any differences in the metabolic and hormonal status of the subjects (intersubject variability) before each trial.

Experimental procedures

Subjects participated in 2 experimental trials separated by at least 10 days and conducted in random order. During each trial, the subjects exercised on a semirecumbent cycle ergometer for 120 minutes. They commenced exercise for 5 minutes at 50% VO_{2max} and subsequently cycled for 115 minutes at ~65% VO_{2max}. Trials were conducted in a room maintained at 22°C, and a circulating fan was placed in front of the subjects during exercise to minimize thermal stress. Each trial was identical except that in one trial (GLU) subjects ingested 250 mL of a 6.4% carbohydrate beverage (Lucozade Sport; Glaxo Smith Kline, UK) at the onset of and at 15 minutes intervals throughout exercise, whereas in the other they consumed an artificially flavored placebo (CON). On the day of each experiment, the subjects reported to the laboratory at 0730 hours after a 12–14 hours overnight fast. They voided, changed into appropriate exercise attire, and rested in a supine position for 10 minutes.

A hepatic venous catheter was subsequently inserted (Febbraio et al 2002a). During experiments on the first 3 subjects, the hepatic venous catheter was introduced via the right median cubital vein and was guided with the subject supine. The position of the catheter was confirmed with fluoroscopy in the body position used during cycling. To ensure that ventilation (V_e) did not displace the catheter, the position was also confirmed after maximal

voluntary V_e. Despite these efforts, the catheter dislodged during exercise in 2 of the 6 experiments, and therefore, we could only obtain data in both trials for 1 subject. Hence, we introduced the catheter via the right femoral vein in the subsequent experiments in the 3 subjects, and in these trials, the catheter remained in the hepatic vein. As a result, data presented for hepatosplanchnic Hsp72-Hsp60 flux are presented as $n = 4$. After this procedure, a catheter was placed in the left brachial artery (1.0 mm inner diameter; 20 gauge) and a third catheter (7 Fr diameter Cook, Denmark) was inserted into the left femoral vein ~1–2 cm distal to the inguinal ligament (Febbraio et al 2002a). Hepatosplanchnic blood flow (HBF) and LBF was measured at rest and at 60 and 120 minutes during exercise using the indocyanine green dye and the constant infusion thermodilution techniques, respectively (Febbraio et al 2002a).

Immediately before exercise and at 60 and 120 minutes during exercise, blood samples were simultaneously collected from the brachial artery and the femoral and hepatic veins for the measurement of Hsp72 and Hsp60 as described previously (Febbraio et al 2002a). Muscle biopsy samples were obtained from the vastus lateralis muscle before and immediately after exercise and analyzed for Hsp72 (#EKS-700, Stressgen Biotechnologies, Victoria, BC, Canada) and Hsp60 (#EKS-600, Stressgen Biotechnologies) proteins by an enzyme-linked immunosorbent assay using methods described in detail elsewhere (Walsh et al 2001). Muscle glycogen was also measured by enzymatic analyses with fluorometric detection as described previously (Febbraio et al 2002c). Care was taken to sample muscle from different regions of the muscle before and after exercise. We have demonstrated previously that this biopsy procedure does not result in increased Hsp72 mRNA in noncontracting muscle (Febbraio et al 2002b).

Calculations and statistics

Net leg Hsp72 and Hsp60 balances were calculated by multiplying the femoral vein-arterial Hsp72 and Hsp60 differences by the net LBF. Similarly, the net hepatosplanchnic Hsp72 and Hsp60 balances were calculated by multiplying the hepatosplanchnic vein-arterial Hsp72 and Hsp60 differences by the net HBF. Comparative data are expressed as means ± SEM. A 2-way (trial × time) analysis of variance with repeated measures on the time factor was used to compute the statistics (Statistica®, Tulsa, OK, USA), for all measures. Significance was accepted with a P value of ≤ 0.05. If analyses revealed a significant interaction, a Newman–Keuls post hoc test was used to locate specific differences.

RESULTS

LBF increased ($P < 0.05$) from 0.54 ± 0.07 and 0.56 ± 0.06 L/min at rest to an average of 6.12 ± 0.52 and 6.14 ± 0.38 L/min during exercise for CON and GLU, respectively. HBF was neither affected by exercise nor glucose ingestion averaging 1.26 ± 0.18 and 1.29 ± 0.20 L/min for CON and GLU, respectively ($n = 4$).

Glucose ingestion attenuates the exercise-induced increase in circulating Hsp72 and Hsp60

The arterial serum Hsp72 response increased ($P < 0.05$) throughout exercise in both GLU and CON; however, the increase during the final 60 minutes of exercise was attenuated ($P < 0.05$) in GLU (Fig 1). The large standard error bars at 120 minutes of exercise when comparing arterial serum Hsp72 values between trials was because of a differential Hsp72 response between subjects rather than between trials because all 6 subjects displayed markedly lower concentrations at 120 minutes in GLU compared with CON. As a result, the difference when comparing trials at this point was highly significant ($P = 0.006$). Arterial serum Hsp60 increased ($P < 0.05$) after 60 minutes of the control trial before returning to resting levels at the end (120 minutes) of exertion. Such increase was totally abolished with glucose ingestion ($P < 0.05$) (Fig 2). The large standard error bars in arterial serum Hsp60 values was because of a high intersubject variability in basal Hsp60 levels rather than different Hsp60 responses with exercise or treatment. In fact, all 6 subjects displayed markedly lower concentrations at 60 minutes in GLU compared with CON. As a result, the difference when comparing trials at this point was highly significant ($P = 0.003$).

The reduction with glucose ingestion of the exercise-induced increase in circulating Hsp72 was because of a blunting in hepatosplanchnic Hsp72 release

To test the hypothesis that the attenuation of the exercise-induced increase in circulating Hsps by glucose ingestion was because of a reduction in the hepatosplanchnic rather than leg Hsps release, we calculated both leg and hepatosplanchnic Hsps releases. There was no measurable leg serum Hsp72 or Hsp60 release in either GLU or CON. Although we were only able to analyze hepatosplanchnic Hsp72 release from 4 subjects, hepatosplanchnic Hsp72 release was markedly attenuated in GLU compared with CON in all subjects (Fig 1). Although we had a low subject number, statistical analyses revealed hepatosplanchnic Hsp72 release in CON ($P < 0.05$) but not in GLU. Therefore, the attenuation of the exercise-induced increase in circulating Hsp72 was in part because of the abolished

hepatosplanchnic Hsp72 release with glucose ingestion. We did not detect hepatosplanchnic Hsp60 release in either trial (Fig 2).

Glucose ingestion does not affect intramuscular Hsp72 and Hsp60 protein expression or muscle glycogen use

Neither muscle Hsp72 nor Hsp60 protein expression was affected by exercise or glucose ingestion (Fig 3). Although Hsp72 appeared elevated before exercise in GLU compared with CON, this was largely because of the results from 1 subject. Hence, even when the data from this subject were included, analysis by a paired *t*-test on the preexercise data results was not significant ($P = 0.13$). Of note, muscle Hsp72 expression was much higher than Hsp60 protein expression. Muscle glycogen content was reduced ($P < 0.05$) during exercise but was not different when comparing GLU with CON either before or after exercise (Fig 3).

DISCUSSION

The results from the present study demonstrate that glucose ingestion during exercise attenuates the exercise-induced increase in serum Hsp72 and Hsp60. However, 120 minutes of moderate intensity cycling does not affect the Hsp72 or Hsp60 protein expression within contracting skeletal muscle.

It has been reported that Hsp60 is expressed in the serum of healthy and hypertensive subjects (Pockley et al 2002), with decreased values in aging (Rea et al 2001). Interestingly, we report in this study that physical exertion induces a small increase in circulating Hsp60 during the first 60 minutes of submaximal exercise, returning to basal values after 120 minutes of exercise. To our knowledge, this is the first report that similar to Hsp72 (Walsh et al 2001; Febbraio et al 2002a), Hsp60 is also induced in humans during exercise. Of note, neither the contracting limb nor the liver contributed to the increase in circulating Hsp60. It appeared that leg and hepatosplanchnic serum Hsp60 release was higher in GLU at 60 minutes, when arterial serum Hsp60 was lower ($P < 0.05$) at this time point compared with CON. However, this was largely because of the results from 1 subject, with all others showing little if any change. This was contrary to the consistently higher arterial serum Hsp60 displayed by all subjects in CON compared with GLU. It is also noteworthy, that the absolute release of Hsp60 peaked at 4 ng/min for the leg and 0.6 ng/min for the hepatosplanchnic viscera, whereas the arterial concentrations ranged between 8 and 12 ng/mL (Fig 2). This is in contrast with the Hsp72 data, where the peak hepatosplanchnic release matched the arterial concentration more closely (Fig 1). Hence, the data suggest that other tissues release Hsp60

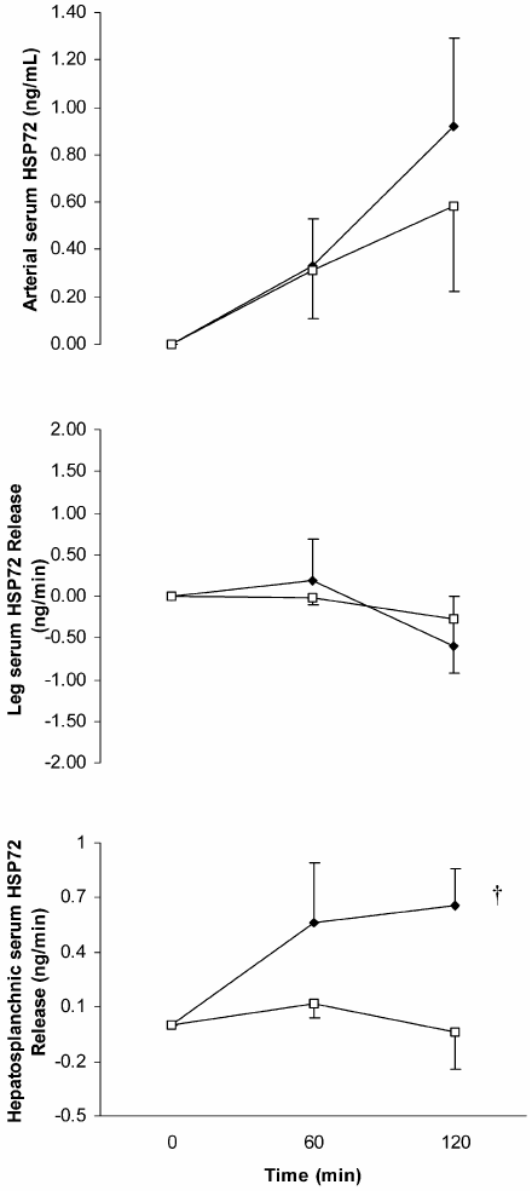

Fig 1. Arterial serum heat shock protein 72 (Hsp72) (top), leg Hsp72 release (middle), and hepatosplanchnic Hsp72 release (bottom) before (0 minute) and during 120 minutes of semirecumbent cycling at ~65% of maximal oxygen uptake with the ingestion of a placebo (CON, filled diamonds) or glucose (GLU, open squares) beverage throughout exercise. * denotes difference when comparing CON with GLU, † denotes main effect for time ($P < 0.05$) in CON. Data expressed as mean ± SEM ($n = 6$ for top and middle panel, $n = 4$ for bottom panel).

in blood during the exercise. In this regard, it has been reported that exercise induces Hsp60 expression in the cytoplasm of leukocytes (Fehrenbach et al 2000), and that hydrogen peroxide, a common oxidant induced by exer-

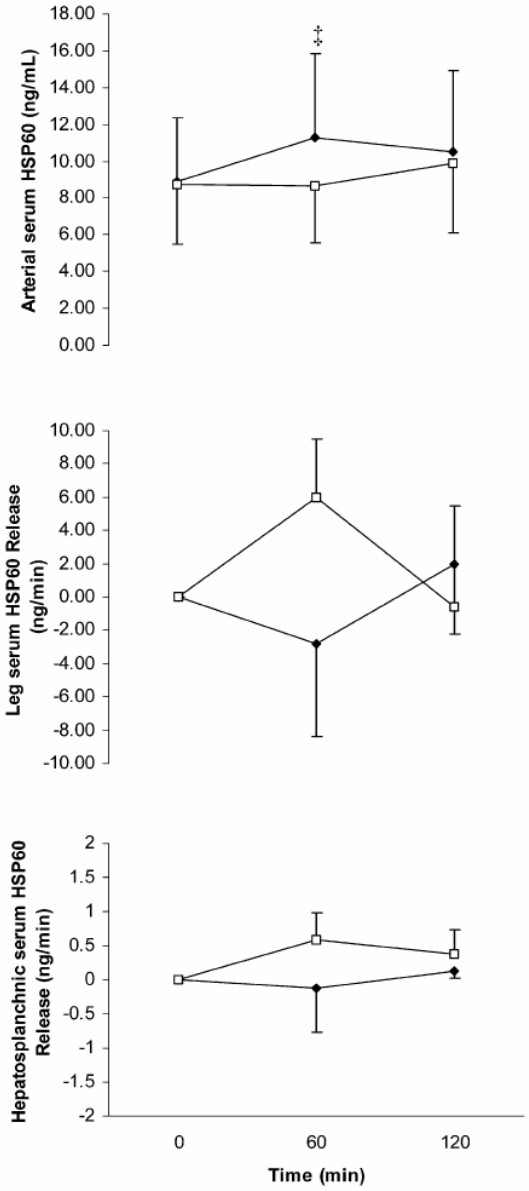

Fig 2. Arterial serum heat shock protein 60 (Hsp60) (top), leg Hsp60 release (middle), and hepatosplanchnic Hsp60 release (bottom) before (0 minute) and during 120 minutes of semirecumbent cycling at ~65% of maximal oxygen uptake with the ingestion of a placebo (CON, filled diamonds) or glucose (GLU, open squares) beverage throughout exercise. ‡ denotes difference when comparing CON with GLU. Data expressed as mean ± SEM (n = 6 for top and middle panel, n = 4 for bottom panel).

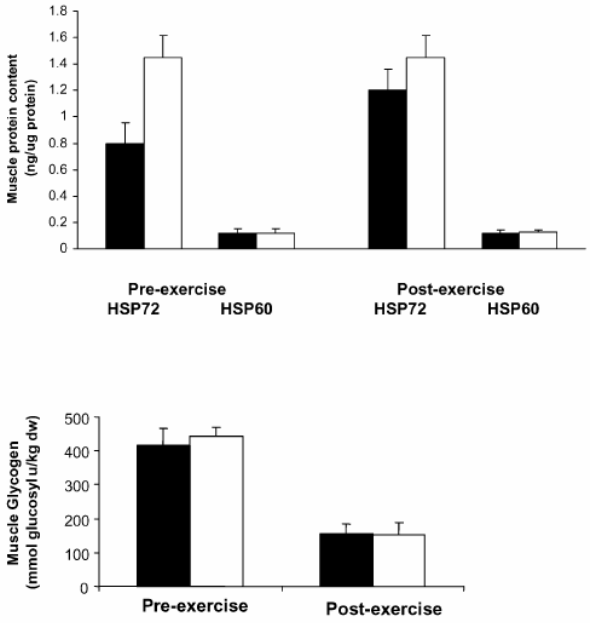

Fig 3. Heat shock protein (Hsp) 72 and Hsp60 in skeletal muscle (top) and glycogen (bottom) obtained before (preexercise) and immediately after 120 minutes of semirecumbent cycling at ~65% of maximal oxygen uptake with the ingestion of a placebo (CON, filled bars) or glucose (GLU, open bars) beverage throughout exercise. Data expressed as mean ± SEM (n = 6).

cise, leads to Hsp60 expression in lymphocytes (Khassaf et al 2003). Furthermore, Hsp60 proteins have been found at unexpected locations, such as the lymphocyte cell surface (Soltys and Gupta 1996; Khan et al 1998), suggesting the possibility that they may be released into the blood (Soltys and Gupta 1999). Therefore, our data suggest that rather than contracting muscle fibers or hepatocytes, other cells may contribute to the exercise-induced increase in circulating Hsp60, which is quickly returned to basal levels after 120 minutes of exercise, denoting a rapid Hsp60 turnover.

We show that glucose ingestion attenuates the exercise-induced increase in circulating Hsp60, suggesting that Hsp60 is released into or expressed in the blood (by other tissues than contracting limb or liver) in absence of exogenous glucose, when the hepatic glucose production is increased (Jeukendrup et al 1999a). Whether there is any causal relationship between liver glycogenolysis or gluconeogenesis (or both) and circulating Hsp60 remains to be elucidated. Because fat oxidation is increased in absence of exogenous glucose (Coyle et al 1997; Angus et al 2000; Spriet and Watt 2003), another question to be answered is whether there is any causal relationship between serum Hsp60 levels and fat oxidation. Further experiments in isolated hepatocytes, myocytes, and adipocytes are required to answer these questions.

It has recently been reported that stress such as physical trauma (Pittet et al 2002) and exercise (Walsh et al 2001; Febbraio et al 2002a) increases the serum levels of Hsp72 in humans. Furthermore, during exercise, the hepatosplanchnic viscera can account for part of this in-

crease (Febbraio et al 2002a), whereas the contracting limb does not contribute to the increase in circulating Hsp72 (Febbraio et al 2002a, 2002c). The results from the present study demonstrate that glucose ingestion can attenuate the exercise-induced increase in arterial serum Hsp72 (Fig 2). It appears that this is at least in part because of a reduction in hepatosplanchnic Hsp72 release. Although we could only collect data in 4 subjects, all 4 demonstrated a total blunting in the hepatosplanchnic Hsp72 release, making it clear that glucose ingestion blunts this response. We are unable to provide a mechanism for such an observation; however, it is possible that the ingestion of glucose reduced hepatic stress because hepatic glucose production during exercise is reduced to basal levels when glucose is ingested (Jeukendrup et al 1999b). Further experiments in isolated hepatocytes are required to provide a mechanism for our observations.

Although hepatosplanchnic serum Hsp72 was totally attenuated in GLU, the arterial systemic Hsp72 was elevated after 120 minutes of exercise (Fig 2). This indicates that tissues other than the hepatosplanchnic viscera contributed to the systemic increase in Hsp72 in this trial. Campisi et al (2003) demonstrated that physical activity increased the Hsp72 content in a number of tissues including the brain, the liver, the heart, the spleen, and the lymph nodes, albeit in a rodent model. In the present study, Hsp72 must have been released from some of these tissues giving rise to the elevated serum Hsp72 response in the presence of a totally attenuated hepatosplanchnic Hsp72 release in GLU. Indeed, we have recently demonstrated that Hsp72 is released from the human brain during 180 minutes of exercise (Lancaster et al 2004).

The observation of a failure for Hsp72 to be increased immediately after an acute bout of exercise supports some (Puntschart et al 1996; Walsh et al 2001) but not all (Febbraio et al 2002c) previous studies. Of note, in the present and previous (Puntschart et al 1996; Walsh et al 2001) studies, the exercise duration ranged from 30 to 120 minutes. In contrast, in our study (Febbraio et al 2002c) showing that a single bout of exercise resulted in an increase in Hsp72 protein expression, such a phenomenon was only observed when exercise was performed for a period of 4–5 hours in the presence of a lower than normal glycogen content at the onset of exercise. When exercise was performed for 4–5 hours with adequate preexercise glycogen stores, no increase in Hsp72 protein was observed (Febbraio et al 2002c). Hence, it appears that only in circumstances where the muscle cells are under a great deal of metabolic stress for prolonged periods does "nondamaging" exercise result in an acute increase in Hsp72 expression.

To our knowledge, only 2 previous studies from the same group (Khassaf et al 2001, 2003) have measured Hsp60 in human skeletal muscle. In these studies, Hsp60 was increased (Khassaf et al 2001) or unaffected (Khassaf et al 2003) 2 days after exercise. In the present study, there was no indication that exercise increased Hsp60 protein expression immediately after exercise, irrespective of glucose ingestion. However, we did observe a much lower basal expression of Hsp60 protein compared with Hsp72. This is not surprising because Hsp72 is cytosolically expressed, whereas Hsp60 resides in the mitochondria. We cannot compare our results with those reported previously (Khassaf et al 2001, 2003) because in these previous studies, the expression of each protein was expressed as a percent change from basal. We were not surprised that glucose ingestion did not affect intramuscular expression of these stress proteins because in this study such a practice did not alter the rate of glycogen use in the contracting muscle. As demonstrated previously, Hsp72 protein expression is influenced by glycogen availability (Febbraio et al 2002c).

In summary, maintaining glucose availability during exercise attenuates the circulating Hsp72-Hsp60 response in healthy humans. The decrease in the Hsp72 systemic response is probably at least in part because of a decrease in the hepatosplanchnic Hsp72 release. Further studies clarifying the role of Hsp72 and Hsp60 in glucose and fat metabolism are warranted.

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A.3. Prolonged interleukin-6 treatment improves glucose tolerance *in vivo* and does not result in activation of the NFκβ signalling pathway

ABSTRACT

Introduction: Cross-sectional studies have associated plasma IL-6 levels with type 2 diabetes, cardiovascular disease, and obesity-related metabolic traits. Conversely, no causal relationships have been published, and plasma IL-6 levels increase considerably after physical exercise, on of the best measures to prevent metabolic complications. Therefore we tested whether IL-6 administration (both acute and chronic) has deleterious effects on insulin signalling and glucose metabolism in rats.

Methods: Male wistar rats (250 g) were treated with saline (CON) or 2.4 μg daily of recombinant rat IL-6 delivered by miniosmotic pump (CHRONIC) or twice daily injection (PHASIC) for 14 days. Animals underwent intraperitoneal glucose tolerance (IPGTT; day 10) and insulin tolerance (IPITT; day 13). On day 14 mice were treated with vehicle (Basal) or 300 ng insulin (INS) and after 5 min muscle and liver tissue were excised and rapidly frozen.

Results: Both CHRONIC and PHASIC treatment markedly enhanced glucose tolerance compared with CON (AUC 79 ± 4 vs 83 ± 9 vs 120 ± 6 mM*min for CHRONIC, PHASIC and CON respectively; $P<0.05$), in the presence of similar plasma insulin levels when comparing the three treatments during the IPGTT. While not significant, insulin tolerance also showed a tendency for improvement with [glucose] 30 min following treatment averaging 1.7 ± 0.3 vs 1.5 ± 0.2 vs 2.0 ± 0.2 mM for CHRONIC, PHASIC and CON respectively. Insulin treatment increased both liver and muscle Akt (Ser^{473}) and STAT3 (Tyr^{705}) phosphorylation, but the level of phosphorylation of these proteins was not different when comparing treatments. Irrespective of mode of delivery, IL-6 treatment did not affect GLUT4, SOCS3, NFκβ, p70 S6K total protein or phosphorylation of IKβ-α ($Ser^{32/36}$) or mTOR (Ser^{2448}) in either muscle or liver.

Conclusions: In summary, irrespective of mode of delivery, 14 days of treatment with IL-6 at 2.4 μg/d results in markedly improved glucose tolerance in healthy rats. This improvement is not, however, associated with enhanced insulin signalling in muscle or liver. Furthermore, the NFκβ/IKK pathway is not activated by chronic administration of IL-6 in either liver or muscle suggesting that IL-6, delivered at this dose, does not activate pro-inflammatory pathways.

Key words: Insulin signalling, glucose metabolism, interleukin-6

A.3.1. Introduction

Accumulating evidence suggest a link between inflammatory markets and metabolic complications. Markers of inflammation, including C-reactive protein (CRP) and the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1, interferon-γ, and IL-6 are elevated in patients with metabolic complications such as cardiovascular disease or type 2 diabetes (Pickup et al., 1997; Pickup & Crook, 1998; Shiba et al., 1998; Kern et al., 2001). Importantly, recent studies demonstrate that levels of CRP and IL-6 correlate with both insulin resistance and obesity and predict the development of type 2 diabetes and cardiovascular disease (Ridker et al., 2000; Pradhan et al., 2001, Spranger et al., 2003; Bluher et al., 2005; Tzoulaki et al., 2005).

Paradoxically aerobic physical exercise, one of the best measures to prevent cardiovascular disease and type 2 diabetes-related metabolic traits, increases acutely plasma and muscle IL-6 levels (Ostrowski et al., 1998; Febbraio & Pedersen, 2002).

In a physiological context, IL-6 levels are maintained in a persistent, chronically elevated state in cardiovascular disease and type 2 diabetes (Bluher et al., 2005; Tzoulaki et al., 2005). However, after physical exercise, IL-6 is released acutely, in a phasic way. Therefore, in order to clarify the role of IL-6, in the current study we have examined the effects of both acute and chronic IL-6 exposure on insulin sensitivity and glucose metabolism in rats. To mimic the constant elevated IL-6 of cardiovascular disease, type 2 diabetes and obesity, we have used implantable osmotic pumps that deliver IL-6 continuously to rats over 14 days. To mimic the exercise-induced IL-6, we injected rats with recombinant rat IL-6 daily, for 14 days. We have examined the impact of both chronic and phasic IL-6 exposure on glucose metabolism, and on downstream insulin action in the liver and muscle.

A.3.2. Methods

Antibodies and reagents. Antiphosphotyrosine antibody (4G10), polyclonal rabbit anti-p85, anti–phosphoAKT (Ser^{473}), anti-phospho-mTOR (Ser^{2448}), and anti-phospho IKβ-α ($Ser^{32/36}$) were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-STAT3, anti-phosphoSTAT3 (Tyr^{705}), and antibodies for GLUT4, SOCS3, NFκβ, and p70 S6K were purchased from Cell Signaling Technology (Beverly, MA). Recombinant rat IL-6 was purchased from RDI systems (Flanders, NJ). All other chemicals were from Sigma except where indicated differently.

Animal use. Male Wistar rats (250 g) were used in all studies. The age of animals used in the study ranged from 8–14 weeks. All animal procedures were in accordance with the Royal Melbourne Institute of Technology animal care guidelines and approved by the animal care and use committee.

Acute IL-6 treatments. Rats were intraperitoneally injected, twice (7am and 7pm) daily with either 1.2 μg of recombinant rat IL-6 (Phasic group) or 1.2 μg of saline infusion (Control group).

Chronic IL-6 treatments. For all experiments examining chronic IL-6 exposure, Alzet osmotic pumps (model 2ML2; Durect, Cupertino, CA) with a 14-day pumping capacity and an infusion rate of 0.1μg of IL-6 per hour were used. Pumps were filled to capacity with either saline (Control group) or recombinant rat IL-6 diluted in carrier (0.9% NaCl and 0.1% BSA) (Chronic group). Following induction of sodium pentobarbital general anesthesia, pumps were implanted into the intrascapular subcutaneous space. Incisions were closed with interrupted absorbable sutures.

Glucose tolerance test. A glucose tolerance test was conducted on day 10. Rats were overnight fasted before test. Glucose (1.5 g/kg body weight) was intraperitoneally injected. Blood glucose levels were determined at indicated intervals (5, 15, 30, 60, 90, and 120 min) using an Accu-Chek (Roche Indianapolis, IN) glucose meter and test strips on tail bleeds of awake rats.

Insulin tolerance test. Rats were overnight fasted before test. Insulin (Novagen, Madison, WI) diluted in carrier (0.9% NaCl, 0.1% BSA) was injected intraperitoneally at 0.7 units/kg. Blood glucose levels were determined at indicated intervals (5, 15, 30, 60, 90, and 120 min) using an Accu-Chek (Roche Indianapolis, IN) glucose meter and test strips on tail bleeds of awake rats.

Insulin treatment and tissue recovery. For liver/muscle comparisons, on day 14 rats were treated intraperitoneally with vehicle (Basal group) or 300 ng of insulin. Liver and soleus were harvested at 5 min after intraperitoneal injection and frozen in liquid N2 within 15 s.

Homogenization and preparation of extracts. Frozen liver and muscle were homogenized in 16 volumes (weight/volume) (liver) or 10 volumes (muscle) of lysis buffer. Frozen tissues were homogenized using the Brinkman PT 10/35 Polytron. Extracts were kept ice-cold at all times. Extracts were cleared by microcentrifugation at 15,000*g* for 10 min at 4°C.

Immunoblotting. Total protein extracts were subjected to 10% SDS-PAGE, transferred to PDF-membranes, and incubated with primary antibodies according to standard immunoblotting procedures. Extracts were resolved by SDS-PAGE and transferred to

nitrocellulose. Proteins were detected by immunoblotting and visualized using enhanced chemiluminescence (Amersham-Pharmacia).

Statistics. Densitometric scanning was performed on a Gel Doc gel documentation

system (BioRad). Data were analyzed using Quantity One Software (BioRad). Statistical analysis was performed using the Student's *t* test for independent samples. $P<0.05$ was considered statistically significant

A.3.3. Results

All rats finished the experiments successfully, and no differences in weight among groups were found.

Both Chronic and Phasic treatments markedly enhanced glucose tolerance compared with controls (AUC 79 ± 4 vs 83 ± 9 vs 120 ± 6 mM*min for Chronic, Phasic and controls respectively; $P<0.05$), in the presence of similar plasma insulin levels when comparing the three treatments during the glucose tolerance test (Figure 1).

While not significant, insulin tolerance also showed a tendency for improvement with [glucose] 30 min following treatment averaging 1.7 ± 0.3 vs 1.5 ± 0.2 vs 2.0 ± 0.2 mM for Chronic, Phasic and controls respectively.

Insulin treatment increased both liver and muscle Akt (Ser^{473}) and STAT3 (Tyr^{705}) phosphorylation, but the level of phosphorylation of these proteins was not different when comparing treatments (Figures 2, 3). Irrespective of mode of delivery, IL-6 treatment did not affect GLUT4, SOCS3, NFκβ, p70 S6K total protein (Figure 4, 5) or phosphorylation of IKβ-α ($Ser^{32/36}$) or mTOR (Ser^{2448}) in either muscle or liver (Figures 6, 7).

A.3.4. Discussion

Our data suggest that, irrespective of mode of delivery, 14 days of treatment with IL-6 at 2.4 μg/d results in markedly improved glucose tolerance in healthy rats. This improvement is not, however, associated with enhanced insulin signalling in muscle or liver. Furthermore, the NFκβ/IKK pathway is not activated by chronic administration of IL-6 in either liver or muscle suggesting that IL-6, delivered at this dose, does not activate pro-inflammatory pathways.

There is evidence showing associations between plasma IL-6 levels and risk of type 2 diabetes, cardiovascular disease, or related metabolic traits (Ridker et al., 2000; Pradhan et al., 2001, Spranger et al., 2003; Bluher et al., 2005; Tzoulaki et al., 2005). These studies apparently are contradictory with our data in the present study. Critically, however, there are no studies providing any evidence that elevated IL-6 actually cause insulin resistance, type 2 diabetes or cardiovascular disease through a specific pathway. Given that much of the IL-6 is produced by adipose tissue at rest (Mohamed-Ali et al., 1997), it is possible that the associations between IL-6 and insulin sensitivity seen in individuals with obesity-related traits is the result of elevated adiposity in these individuals, rather than IL-6 *per se.* Indeed, Vozarova et al. (2001) demonstrated that in an ethnic population susceptible to insulin resistance, IL-6 was negatively correlated to insulin action and positively correlated to adiposity. However, after adjustment for adiposity, there was no correlation between IL-6 and insulin action. Carey et al. (2004) conducted a study in which they measured BMI and IL-6 in patients with Type 2 diabetes and in healthy matched control subjects. They found no relationship between IL-6 and insulin sensitivity, but they found a strong relationship (r=0.85; p<0.00001) between IL-6 and BMI. On balance, therefore, we would suggest that IL-6 is indeed associated with insulin resistance, but that this association is more likely to be due to the elevated fat mass characteristic of individuals with obesity-related metabolic traits, rather than IL-6 *per se*.

Far from finding associations between IL-6 and metabolic traits, we found that 14 days of treatment with IL-6 at 2.4 μg/d results in markedly improved glucose tolerance in healthy rats. This enhancement was irrespective of mode of delivery (chronic or phasic), but was not associated with enhanced insulin signalling in muscle or liver. In fact, downstream insulin signalling was not enhanced with IL-6, and the protein content of GLUT4 was not affected. Therefore, our data suggest that the IL-6-induced enhancement in glucose tolerance should be due to other insulin-independent pathways. More studies are required to clarify the possible mechanisms by which IL-6 can improve glucose tolerance.

Another interesting point in our study is that IL-6 administration did not activate inflammatory pathways (e.g., NFκβ), suggesting that associations found in cross-sectional studies between plasma IL-6 and inflammatory related markers or related metabolic traits (Ridker et al., 2000; Pradhan et al., 2001, Spranger et al., 2003; Bluher et al., 2005; Tzoulaki et al., 2005) are not causal.

On summary, our data suggest that, irrespective of mode of delivery, 14 days of treatment with IL-6 at 2.4 μg/d results in markedly improved glucose tolerance in healthy rats, and does not activate pro-inflammatory pathways. Therefore, IL-6 might be considered as a

molecule responsible, at least in part, for exercise-induced metabolic improvement. Further studies in humans are warranted to test this hypothesis.

A.3.5. References

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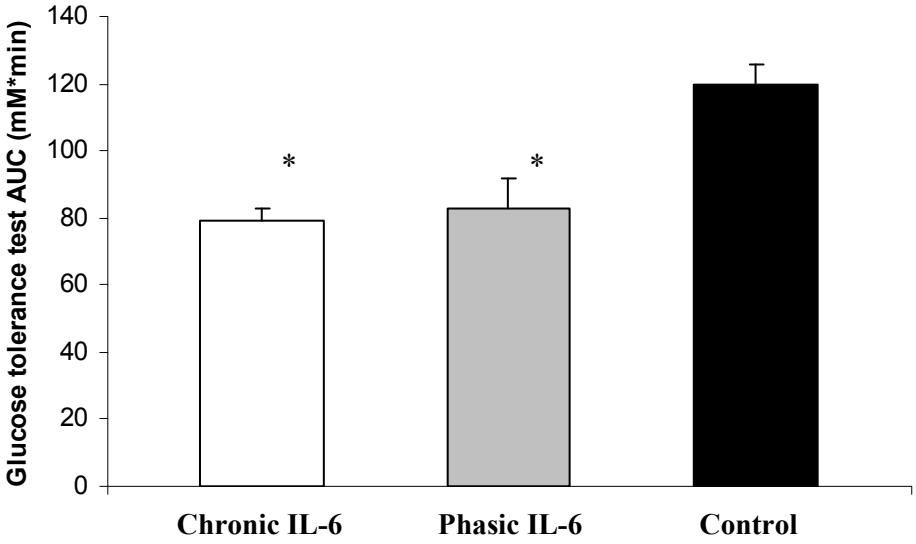

Figure 1. Area under the curve (AUC) of plasma glucose levels during a 2-h glucose tolerance test in healthy rats after chronic IL-6, phasic IL-6, or saline administration during 10 days. Values are expressed as means (bars) and standard deviations (error bars). * denotes significant differences ($P<0.05$) compared with controls.

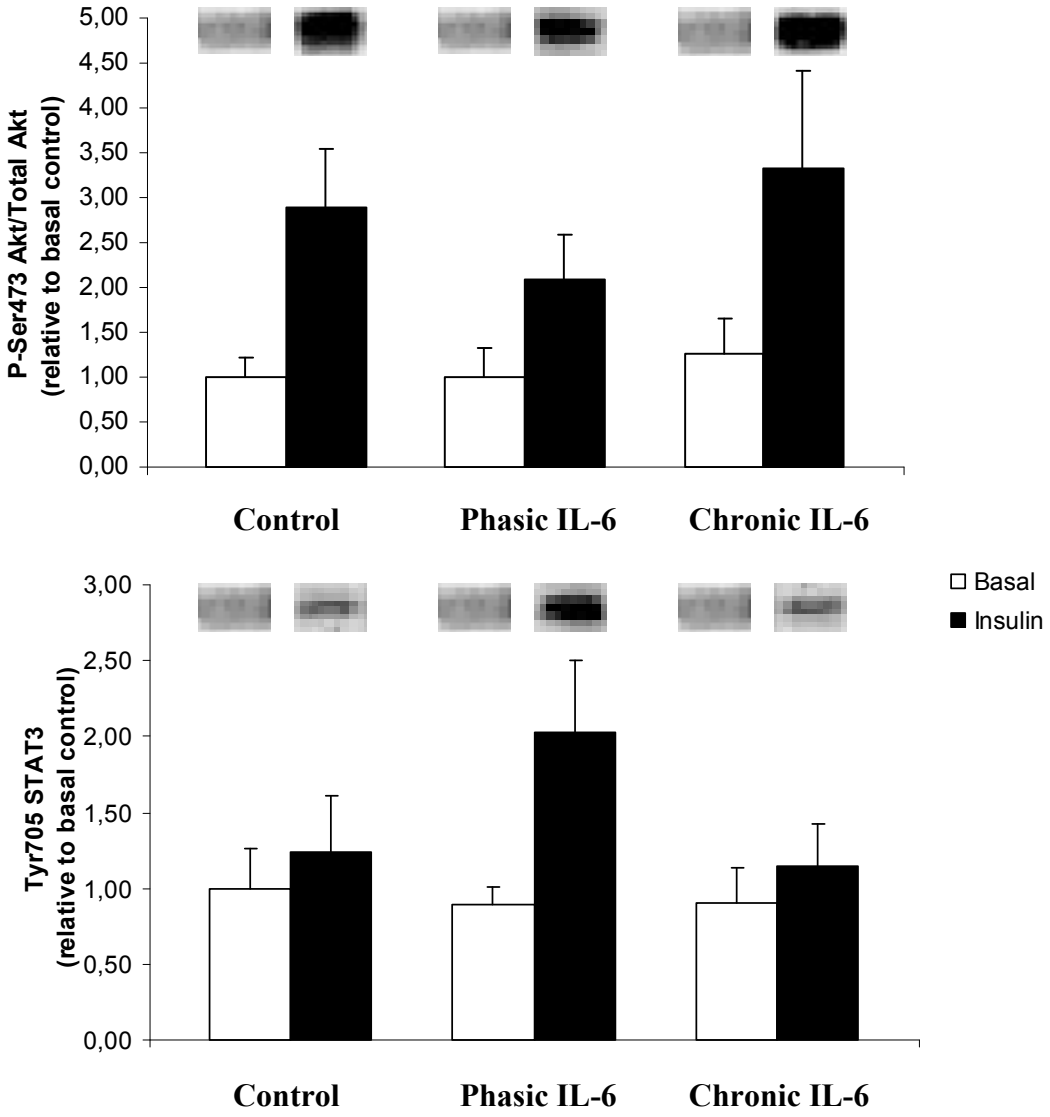

Figure 2. Both Akt and STAT3 are activated in skeletal muscle (soleus) after insulin treatment, with no difference among groups (Chronic IL-6, Phasic IL-6, or saline administration). Western blot analysis was performed to assess Akt and STAT3 activation in muscle homogenates using anti-Ser473 Akt antibody and anti-Tyr705 STAT3 antibody. Values are means (bars) and standard deviations (error bars).

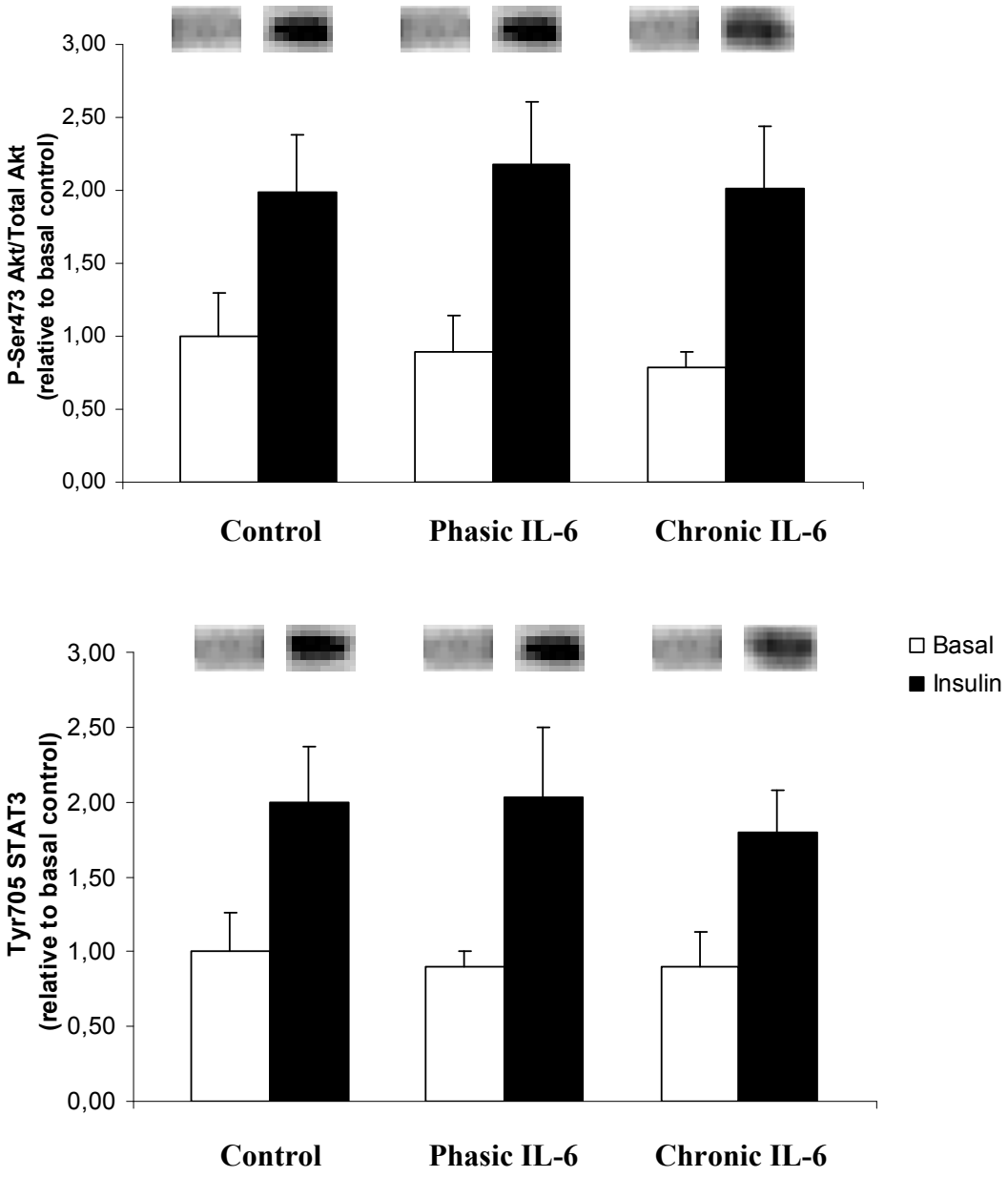

Figure 3. Both Akt and STAT3 are activated in liver after insulin treatment, with no difference among groups (Chronic IL-6, Phasic IL-6, or saline administration). Western blot analysis was performed to assess Akt and STAT3 activation in liver homogenates using anti-Ser473 Akt antibody and anti-Tyr705 STAT3 antibody. Values are means (bars) and standard deviations (error bars).

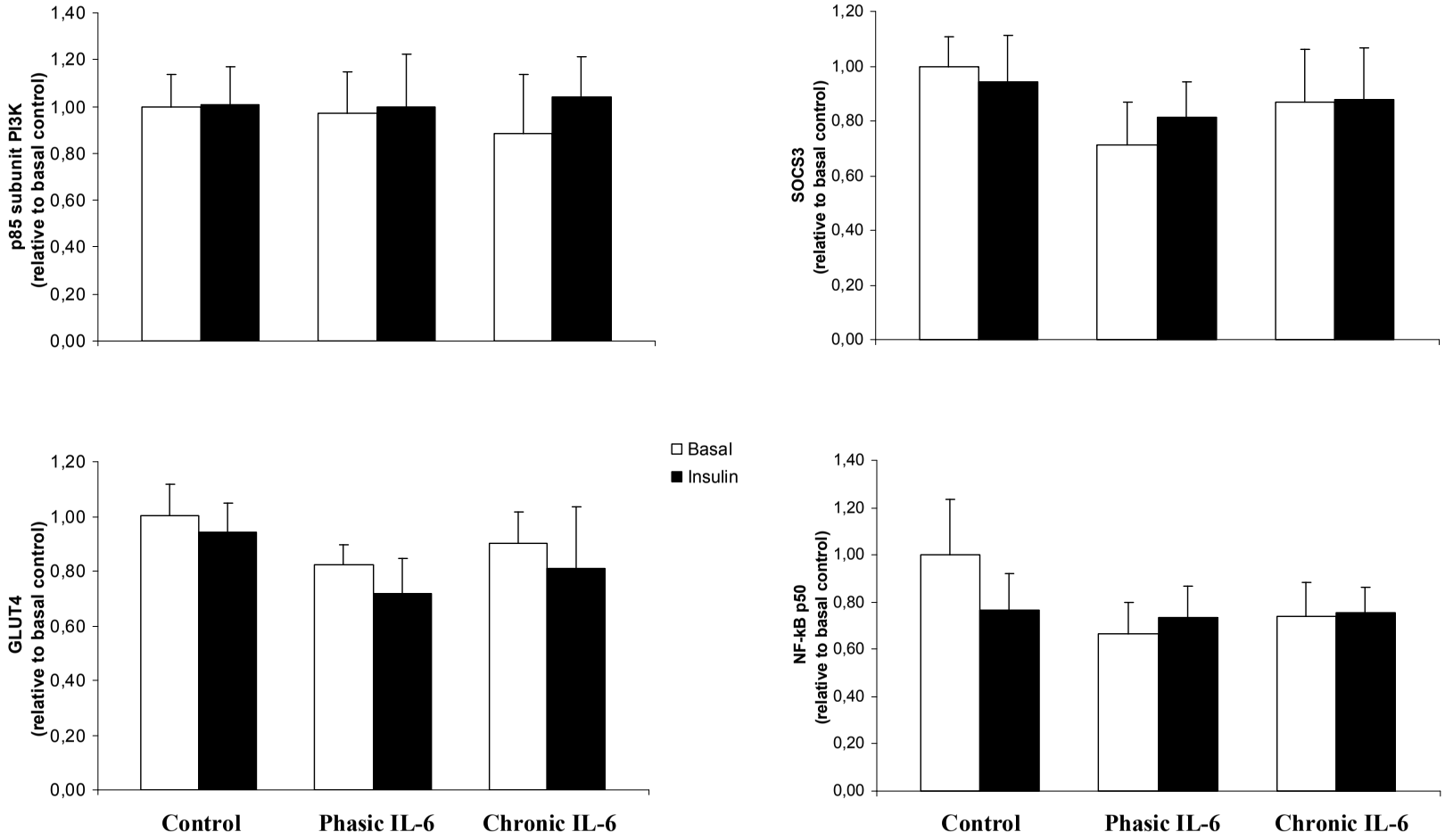

Figure 4. Protein content of specific proteins related to insulin signalling pathways in skeletal muscle (soleus). Values are expressed as means (bars) and standard deviations (error bars). No differences were found among groups.

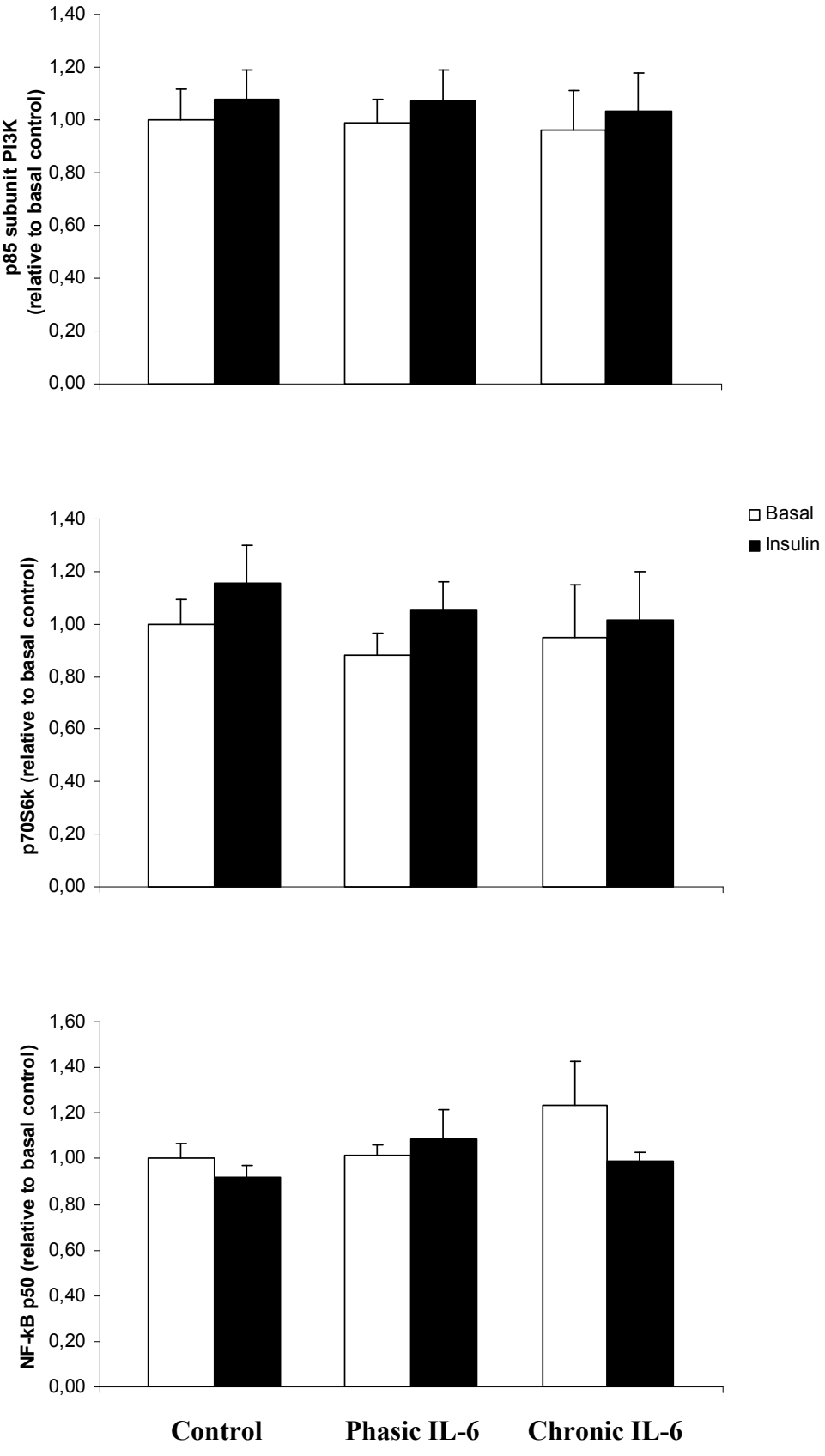

Figure 5. Protein content of specific proteins related to insulin signalling pathways in liver. Values are expressed as means (bars) and standard deviations (error bars). No differences were found among groups.

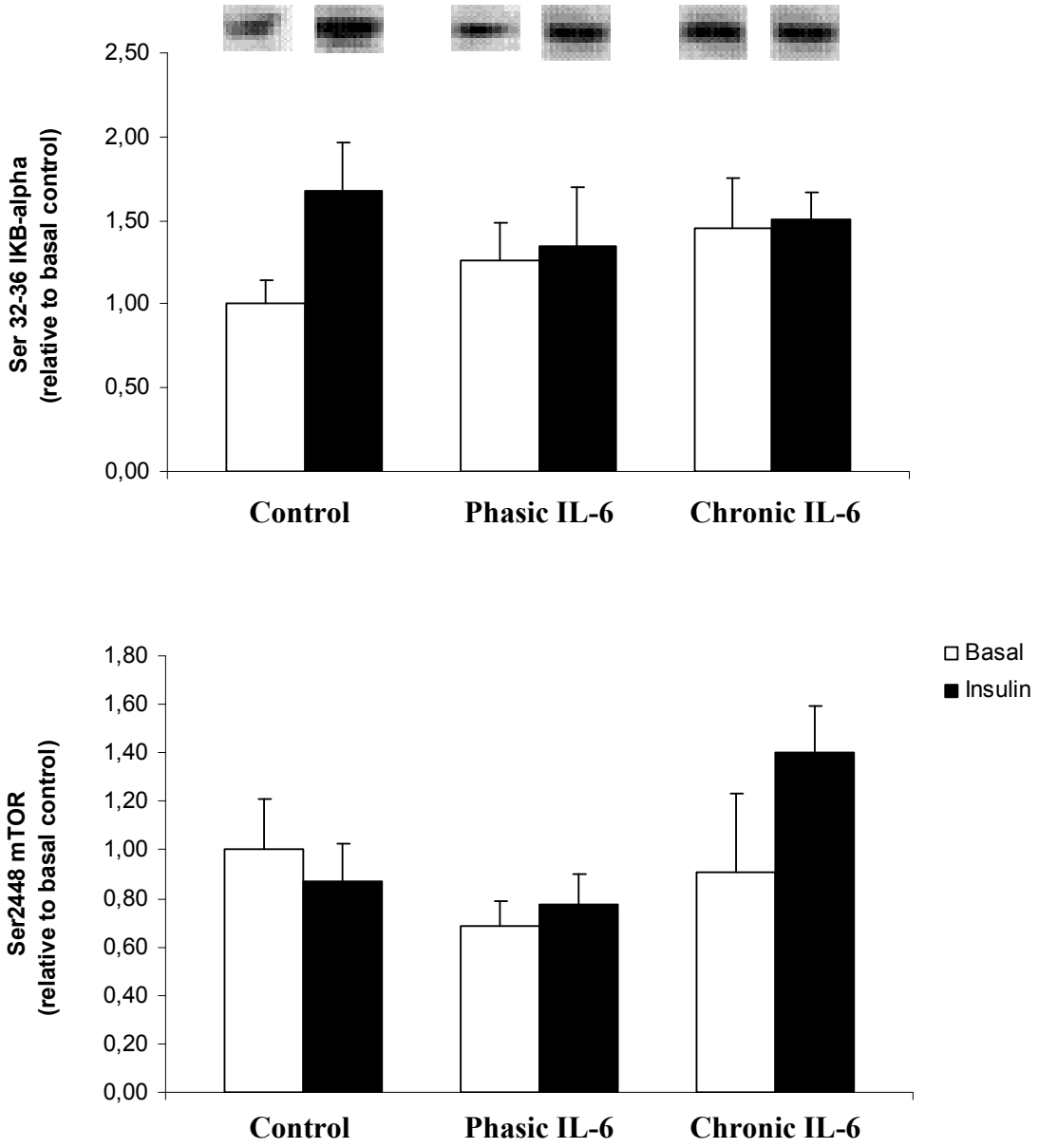

Figure 6. Both IKB and mTOR are not activated in skeletal muscle after insulin treatment, with no difference among groups (Chronic IL-6, Phasic IL-6, or saline administration). Western blot analysis was performed to assess IKB and mTOR activation in muscle homogenates using anti-Ser32-36 IKB antibody and anti-Ser2448 mTOR antibody. Values are means (bars) and standard deviations (error bars).

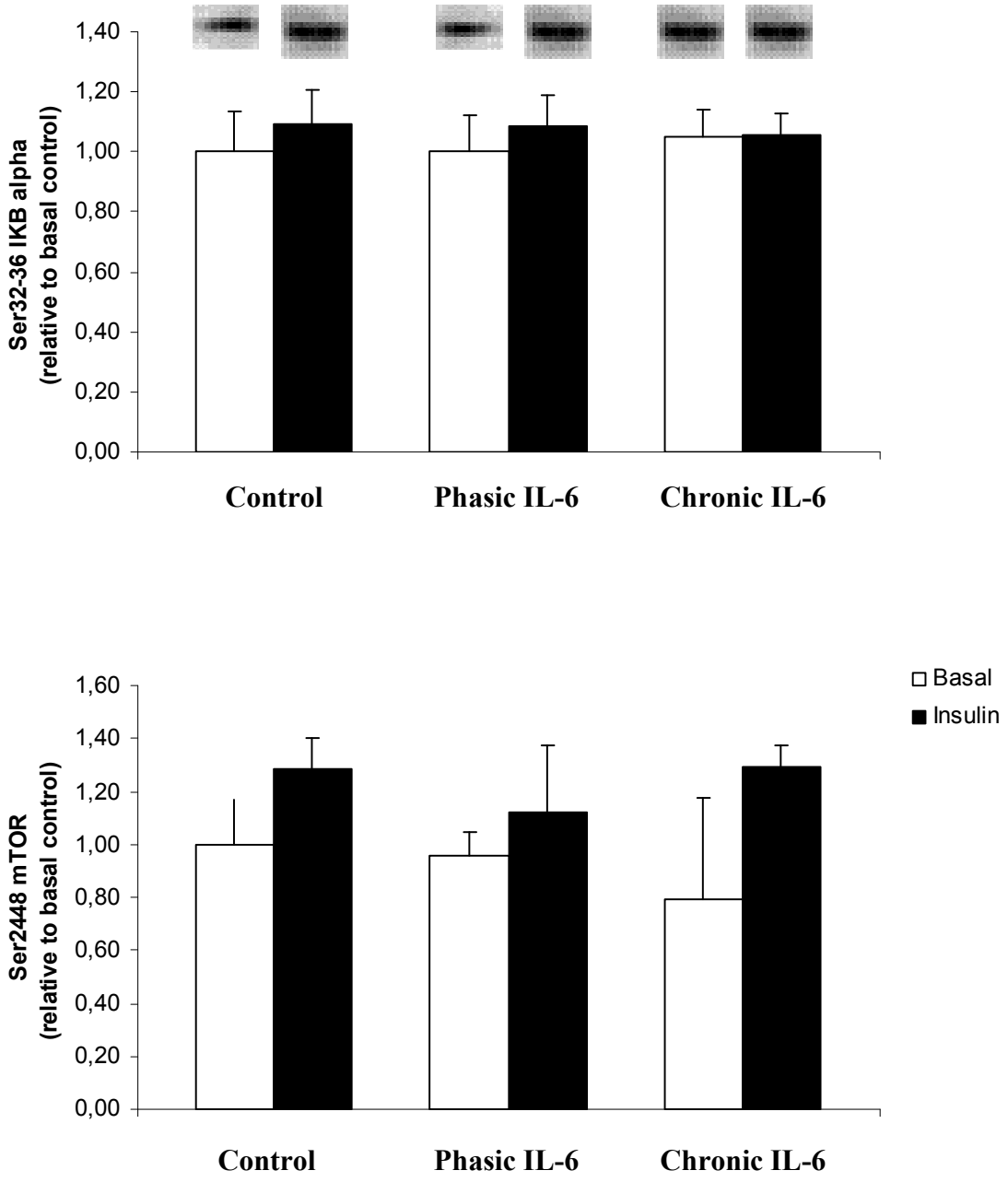

Figure 7. Both IKB and mTOR are not activated in liver after insulin treatment, with no difference among groups (Chronic IL-6, Phasic IL-6, or saline administration). Western blot analysis was performed to assess IKB and mTOR activation in liver homogenates using anti-Ser32-36 IKB antibody and anti-Ser2448 mTOR antibody. Values are means (bars) and standard deviations (error bars).

A.4. ANNEX Conclusions

Exercise-induced HSPs

We demonstrate in the annex of this PhD Thesis that plasma HSP60 and HSP70 increase with physical exercise. In relation to any possible biological role of this increase, there are some studies suggesting beneficial metabolic effects. Plasma HSPs levels have been reported to protect from atherosclerosis (Pockley et al., 2003). Another study has reported Hsp70 levels to be lower in patients at the time of diagnosis of coronary artery disease by coronary angiography (Zhu et al., 2003). In Zhu et al's study, individuals exhibiting Hsp70 levels below the median had twice the risk of coronary artery disease than individuals with levels above the median, and disease severity (number of diseased vessels) was also inversely associated with circulating Hsp70 levels. One study has addressed this issue and demonstrated that extracellular Hsp70 protects stressed aortic cells in culture by a mechanism that appears to involve cell surface binding (Johnson et al., 1990). Another mechanism by which Hsp70 might modify the establishment and/or progression of atherosclerosis is via an anti-inflammatory effect. Intracellular Hsp70 has been shown to attenuate inflammatory responses, because elevating intracellular levels of Hsp70 in the vasculature reduces leukocyte adhesion at inflammatory sites (House et al., 2001). Taken these data together, we can conclude that the exercise-induced increase in HSP levels may mediate beneficial metabolic effects. Future studies are warranted to confirm this possibility.

Exercise-induced IL-6

There are some studies showing associations between plasma IL-6 levels and risk of type 2 diabetes, cardiovascular disease, or related metabolic traits (Ridker et al., 2000; Pradhan et al., 2001, Spranger et al., 2003; Bluher et al., 2005; Tzoulaki et al., 2005). Paradoxically aerobic physical exercise, one of the best measures to prevent cardiovascular disease and type 2 diabetes-related metabolic traits, increases acutely plasma and muscle IL-6 levels (Ostrowski et al., 1998; Febbraio & Pedersen, 2002).

In this PhD Thesis we intended to mimic exercise-induce IL-6 increae, by administrating IL-6 either acutely or chronically. In both cases IL-6 administration improved considerably glucose

tolerance in animal models, and did not activate pro-inflammatory pathways. Other experiments *in vitro* have been performed, confirming our results and showing that IL-6 may increase glucose uptake (Febbraio et al., unpublished data). In addition, a study conducted in humans has shown that IL-6 may activate lipolysis independently of elevations in growth hormone and/or cortisol and appears to be a potent catalyst for fat oxidation in muscle cells (Petersen et al., 2005). These data, taken with caution, suggest that IL-6 might be considered as a molecule responsible, at least in part, for exercise-induced metabolic improvement. Further studies are warranted to test this hypothesis.

Although there are studies showing associations between plasma IL-6 and diabetes-relates metabolic traits, critically, however, there are no studies providing any evidence that elevated IL-6 actually cause insulin resistance, type 2 diabetes or cardiovascular disease through a specific pathway. Given that much of the IL-6 is produced by adipose tissue at rest (Mohamed-Ali et al., 1997), it is possible that the associations between IL-6 and insulin sensitivity seen in individuals with obesity-related traits is the result of elevated adiposity in these individuals, rather than IL-6 *per se* (Vozarova et al., 2001; Carey et al., 2004). Another possibility is that adipose- and muscle-derived IL-6 present different biological roles. Further studies are needed to clarify these questions.

In summary, we provide evidence for the possibility that both HSPs and IL-6 may be mediators, at least in part, of the exercise-induced beneficial metabolic effects.

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