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Identification of genetic polymorphisms for antioxidant defense system genes and study of their association with obesity and metabolic syndrome features in children

Identificación de polimorfismos génicos relacionados con el sistema de defensa antioxidante implicados en la obesidad y el síndrome metabólico en niños

Tesis Doctoral con Mención Internacional presentada por:

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SUMMARY

Introduction

According to the World Health Organization childhood obesity is nowadays a global problem affecting a high number of low and middle-income countries, and having a big impact for healthcare systems worldwide (Gupta *et al.* 2012; Han *et al.* 2010).

Obesity is a complex trait that stems from a complicated network of contributory components, including genomic and environmental factors, the aggregations of which increase the probability of disease. Studies in twins, no-twin siblings, and adoptees have estimated that genetic components contribute from 40% to 70% to the inter-individual variation in common obesity (Elks *et al*. 2012). It should also be emphasized that heritability estimates have been shown to increase from early childhood through adolescence (Dubois *et al*. 2012). Genome-wide and candidate gene association studies have successfully revealed a variety of genetic loci associated with the more common form of obesity (reviewed by Rankinen *et al.* 2006 and Xia & Grant 2013).

Obesity is characterized by an excessive accumulation of fat in adipose tissue, which is accompanied by low-grade inflammation, adipokine secretion dysregulation, hypoxia and oxidative stress (Fernández-Sánchez *et al*. 2011). Oxidative stress is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism. The link between obesity and enhanced oxidative stress might be due to the hyperglycemia, high circulating free fatty acids, decreased antioxidant defenses and chronic inflammation associated with obesity (Bondia-Pons *et al*. 2012, Furukawa *et al*. 2004).

The antioxidant defense system maintains ROS homeostasis in the organism. It comprises both endogenous and exogenous antioxidants. Endogenous antioxidants primarily include enzymes such as glutathione peroxidases (GPXs), catalase (CAT), paraoxonases (PONs), superoxide dismutases (SODs) and peroxiredoxins (PRDXs). These enzymes degrade ROS at different levels and in different compartments inside and outside of the cells.

It is known that the presence of single nucleotide polymorphisms (SNPs) can affect the functioning of antioxidant enzymes and increase the risk of certain diseases, such as cancer (Da Costa *et al*. 2012). However, to our knowledge, the impact of genetic variations in the genes associated with oxidative stress has not

been fully studied within the context of obesity. Detailed studies in this field could clarify the mechanisms involved in the development of the comorbidities of obesity, such as metabolic syndrome and insulin resistance.

Rationale and aims of the study

The research group CTS-461 "Biochemistry of Nutrition. Therapeutical implications" focuses one of its lines of work on childhood obesity through different approaches including the study of novel obesity biomarkers, oxidative stress markers and genetic variation analyses through candidate gene association studies. The first approach in the study of the association of genetic variants with childhood obesity was to observe SNPs from obesity-related genes such as 11β-hydroxysteroid dehydrogenase type 1 (*HSD11B1*), neuropeptide Y (*NPY*), fat mass and obesity (*FTO*), among others. These genes were selected within those previously found by our group to be overexpressed in visceral adipose tissue of chidren at prepubertal age or those described in the literature as associated to fat mass. Arising from this study, two articles have been published describing the association of SNP rs3753519 from the *HSD11B1* gene (Appendix I) and variants rs16147 and rs16131 in the *NPY* gene (Appendix II) with higher childhood obesity risk.

The aim of the present thesis was to identify genetic variants of genes related to the antioxidant defense system that affect the risk of obesity in Spanish prepubertal children, as well as to study their impact on oxidative stress status and obesity metabolic complications such as insulin resistance, inflammation and endothelial damage.

Methodology

The present study is a case-control, cross-sectional, multicentre study that included a total of 193 obese (104 boys and 89 girls) and 191 normal-weight (110 boys and 81 girls) children recruited from primary care centers at three Spanish hospitals: University Hospital "Reina Sofía" and Hospital "Valle de los Pedroches", both in Córdoba; and Clinical University Hospital of Santiago de Compostela. The study followed the Helsinki Declaration for Clinical Studies and permissions were obtained from the Ethics Committee of all Centers.

Blood pressure and anthropometric measurements were performed and blood samples were drawn after overnight fasting. A general biochemical analysis was run at the participating hospitals. Plasma adipokines and biomarkers of

inflammation and endothelial damage were measured by Luminex 200 equipment using XMap technology. In addition, oxidized low-density lipoprotein (ox-LDL) and fatty acid-binding protein 4 (FABP-4) were measured by ELISA and high-sensitivity C-reactive protein (hsCRP) was determined with a turbidimetric assay. The vitamins retinol, α-tocopherol and β-carotene plasma levels were analyzed by high-pressure liquid chromatography.

The antioxidant defense system was evaluated by assessing the total antioxidant capacity of plasma (TAC), the activity of the antioxidant enzymes in erythrocytes [CAT, SOD, glutathione reductase (GR) and GPX] and serum paraoxonase 1 (PON1) activities measured with different substrates (paraoxon, phenylacetate, dihydrocoumarin and diazoxon).

A list of 47 candidate genes related with oxidative stress was prepared and 492 SNPs were selected by using the National Center for Biotechnology Information database (dbSNP). Tag SNP selection was done with the HapMap database. For genotyping, the Illumina GoldenGate technology was used. After obtaining the raw results, quality control steps were followed to remove poor quality data. Then, a logistic regression analysis adjusted by sex and age was conducted to study the association of each SNP with the risk of childhood obesity. In the same way, a linear regression model adjusted by sex and age was applied to study the association between the SNPs and the studied phenotypes. An additional adjustment by body mass index (BMI) was conducted in order to better characterize the observed associations. PLINK 1.07 software was used for the bioinformatic analyses.

Results

Anthropometry and biomarker analysis

Obese children showed significantly higher weight, BMI, BMI *z*-score and waist circumference. Systolic and diastolic blood pressures were also significantly increased with obesity in these children. Fasting insulin and homeostasis model assessment for insulin resistance index were significantly higher and quantitative insulin sensitivity check index was significantly lower in obese children, whereas fasting glucose values did not differ between obese and normal-weight children. Total cholesterol, high-density lipoprotein cholesterol and apolipoprotein A-I were significantly lower in obese children. On the contrary, triacylclycerols, apolipoprotein B and FABP-4 were higher in the obese compared to normal-weight children. Low-density lipoprotein was not significantly different between

groups. Fasting plasma concentrations of resistin and leptin were significantly higher in obese than in normal-weight subjects, by contrast decreased levels of adiponectin were found in obese. Myeloperoxidase, active and total plasminogen activator inhibitor 1 were significantly higher in the obese group compared to the normal-weight group, whereas matrix metallopeptidase 9 was not. HsCRP, interleukin 6, interleukin 8 and tumor necrosis factor alpha (TNF-α) were significantly higher in the obese group compared to the normal-weight group, whereas monocyte chemoattractant protein 1 (MCP-1) concentration did not show any difference between groups. Liver transaminases alanine aminotransferase and gamma-glutamil transferase were significantly higher in obese children, whereas aspartate aminotransferase was lower.

Concerning oxidative stress status, ox-LDL was not significantly different between the groups, whereas TAC was significantly lower in obese children. CAT activity in erythrocytes was found to be significantly lower in obese children, but no differences were observed between erythrocyte enzymatic activities of GR, GPX, SOD or PON1 between obese and normal-weight children. The analyzed vitamins α-tocopherol and β-carotene were significantly lower in obese children, with no differences found in retinol between groups.

Genotyping and association analyses

The association analysis showed that 19 SNPs out of the total of 492 analyzed were associated with childhood obesity. A total of 7 SNPs increased childhood obesity risk, whereas 12 SNPs conferred protection against it. The SNPs found are located in the following genes: paraoxonase 1 (*PON1*), catalase (*CAT*), glutathione peroxidases 4 (*GPX4*), 5 (*GPX5*) and 6 (*GPX6*), aldehyde oxidase 1 (*AOX1*), peroxiredoxin 5 (*PRDX5*), oxidation resistance 1 (*OXR1*), oxidative stress responsive 1 (*OXSR1*) and sirtuin 2 (*SIRT2*).

The main results from the linear regression analysis of SNPs negatively associated with obesity and the studied phenotypes were the following:

- The variant rs854566 in *PON1* gene was associated with lower obesity risk and higher lactonase, arylesterase and diazoxonase activities and lower paraoxonase activity of serum paraoxonase 1 in prepubertal children (Chapter 3; Rupérez *et al.* 2013).
- SNPs rs475043 and rs494024 from the *CAT* gene were associated with a lower childhood obesity risk as seen by their association with lower weight.

- SNPs rs757228, rs3746165 and rs8102188 of the *GPX4* gene were associated with lower childhood obesity risk and lower values of oxidative stress markers.
- The variants rs3731722 and rs1050887 from the *AOX1* gene were associated with lower obesity risk and lower values of leptin in prepubertal children.
- The SNP rs4930698 from the *PRDX5* gene was found to be associated with lower obesity risk and lower values biomarkers of inflammation and endothelial damage in prepubertal children.
- The SNP rs1681887 from the *OXR1* gene was associated with lower obesity risk and lower values insulin resistance and oxidative stress biomarkers in prepubertal children.
- The polymorphism rs4955408 from the *OXSR1* gene was negatively associated with childhood obesity and its phenotypes and with lower insulin resistance, endothelial damage and oxidative stress biomarkers.

The results from the linear regression analysis of SNPs positively associated with obesity and the studied phenotypes are the following:

- Variants from the *CAT* gene promoter rs769214, rs7693316 and rs1049982 were associated with higher childhood obesity risk and obesity phenotypes (Chapter 4; Rupérez *et al.* 2013).
- The variants rs28382586 and rs445870 from the *GPX5* gene and rs406113 and rs11757000 from the *GPX6* gene were associated with higher obesity risk in prepubertal children.

Discussion

The continuing increase in the prevalence of obesity and its associated metabolic disorders such as type 2 diabetes together with the evidence of obesity-induced oxidative stress highlights the need of investigating the molecular mechanisms linking oxidative stress and obesity.

Due to the absence of more information in this field, we were encouraged to carry out this research in the present PhD thesis. We conducted a case-control study in a group of prepubertal obese and normal-weight Spanish children in which we studied the association between SNPs for antioxidant defense system genes and the presence of obesity and metabolic traits involved in insulin resistance, oxidative stress, inflammation and endothelial dysfunction.

The phenotype analysis showed the expected findings concerning obesity-related traits such as weight, BMI, WC, SBP and DBP, which were observed to be increased in prepubertal obese children. Insulin resistance biomarkers, lipid profile, adipokines and biomarkers of inflammation and endothelial damage such as MPO (Olza *et al.* 2012-b) were also found to be altered in prepubertal obesity, contributing to the presence of metabolic syndrome in children (Olza *et al.* 2011). A novel finding was the fact that obese children showed a decreased antioxidant defense as seen by lower erythrocyte CAT activity, lower TAC and lower levels of β-carotene and α-tocopherol (Rupérez *et al*. 2013-b). These findings are in line as those describing higher levels of oxidative stress markers such as malondialdehyde, carbonyl groups and GPX activity and lower levels of GSH in obese children. However, the same study found no differences in β-carotene and α-tocopherol possibly due to the smaller number of subjects included in the study (Codoñer-Franch *et al.* 2010). In contrast, we observed no differences in PON1, GR, GPX or SOD activities or in the oxidative stress marker ox-LDL. The young character of this population could explain the absence of greater alterations in the observed oxidative stress status. Nevertheless, the presence of the mentioned changes in antioxidant defenses could indicate a possible origin of the enhanced oxidative stress implicated in the metabolic complications of obesity.

The analysis of SNPs that can affect the functioning of antioxidant enzymes and thus the risk of diseases such as obesity is a potential approach to elucidate the mechanisms involved in the comorbidities of obesity. Although many studies have been conducted to characterize the association between obesity-related SNPs and childhood obesity (Yu *et al.* 2012; Wheeler *et al.* 2013), the studies of variants in genes of antioxidant defense are almost inexistent. Some of the previous findings include SNPs rs662 (Q192R) and rs854560 (L55M) from the *PON1* gene, which failed to show association with obesity in adolescents (Baráth et al. 2006). In another study the authors showed the Ala16Val SNP from the *SOD2* gene to be associated with obesity in children with non-alcoholic steatohepatitis (El-Koofy et al. 2011). Here, we analyzed 492 SNPs for antioxidant defense genes of which 12 showed protection against obesity and 7 increased risk of obesity in prepubertal children. None of the found SNPs had been previously published regarding their association with obesity.

One of the main findings presented in this thesis is the protective role of the variant rs854566 of the *PON1* gene with respect to obesity (Rupérez *et al.* 2013-a). This SNP also exhibited strong associations with serum PON1 activities, namely

increased diazoxonase, lactonase and arylesterase activities. Despite these results, serum PON1 activities were not found to be significantly different in prepubertal obese children. Nevertheless, we demonstrated that lactonase activity is a reliable indicator of PON1 function and should be used in future studies investigating the role of PON1 in the development of obesity and cardiovascular disease.

In the same way, we described how the SNP rs769214 (-844A/G) of the *CAT* gene promoter was associated with childhood obesity (Rupérez *et al.* 2013-b). Although this variant was not associated with CAT activity, it was associated with FABP-4 levels which could indicate a role of CAT in adipose tissue dysfunction present in obesity.

A variety of SNPs were also found in three GPX genes, *GPX4*, *GPX5* and *GPX6*. In the *GPX4* gene we found the SNPs rs757228, rs3746165 and rs8103188 in the 5'-UTR to be associated with lower obesity risk. Although these variants were associated with obesity markers, as well as with higher TAC, none of them was found to be associated with GPX activity in erythrocytes. Like ways, the SNP rs757228 was not associated with GPX activity in a previous study by Zanon-Moreno *et al*. (2013). Two SNPs from *GPX5* (rs28382586 and rs445870) were positively associated with obesity in children and the presence of the risk alleles of these SNPs was also associated with an increase in obesity related phenotypes. Concerning *GPX6*, two SNPs were found in this gene to be associated with a higher childhood obesity risk, rs406113 and rs11757000. Further studies should be carried out in order to understand the role of these variants on enzyme activity.

Two SNPs (rs3731722 and rs1050887) in the aldehyde oxidase 1 gene (*AOX1*) showed association with lower obesity and with lower BMI, WC, weight and leptin in children. Regarding this variant, there is one previous study were Hartmann *et al.* described how the SNP rs3731722 (also called H1297R) has an impact increasing the metabolizing efficiency of AOX1 (Hartmann *et al*. 2012). Taken together, this could mean that an enhanced detoxifying activity of AOX1 might have an impact on metabolism and a role in the protection against obesity.

In a similar way, we found SNPs rs4930698 (*PRDX5*), rs1681887 (*OXR1*), rs4955408 (*OXSR1*) and rs892034 (*SIRT2* promoter) to be negatively associated with obesity. It is known that SIRT2 mediates an inhibitory effect on adipocyte differentiation (Jing *et al.* 2007). Thus, it could be hypothesized that the presence of rs892034 in the promoter of SIRT2 could increase its expression in adipose

tissue and inhibit differentiation, thus providing a possible explanation of this SNP's function protecting against obesity.

These results must be validated in larger and different populations in order to ensure the quality of the findings. Future *in vivo* and *in vitro* studies on the function of some of these variants in adipose tissue may help us to identify potential antioxidant and protective roles for these enzymes in the protection against the development of obesity and its co-morbidities.

Conclusion

The found SNPs in antioxidant defense system genes have an impact on obesity risk in prepubertal Spanish children. Moreover, the presence of the SNPs influences a variety of phenotypes associated with obesity, insulin resistance, inflammation, endothelial damage and oxidative stress. This finding supports the role of oxidative stress in the pathogenesis of obesity and its derived metabolic complications.

Limitations of the study

Although our population under study was considerably big, a larger group of subjects would have added strength to the observed findings. We did not validate our findings in a different population. The majority of the observed SNPs do not have a clear effect on genetic regulation or in the coded protein, indicating that they are most probably marker SNPs and not causative SNPs. The observed associations failed to remain significant after multiple testing Bonferroni analysis.

RESUMEN GENERAL

Introducción

Según la Organización Mundial de la Salud la obesidad infantil es hoy en día un problema global que afecta a un gran número de países, así como un lastre para los sistemas de salud en todo el mundo (Gupta *et al*. 2012; Han *et al*. 2010).

La obesidad es una enfermedad compleja fruto de la unión de factores genéticos y ambientales, que en su conjunto incrementan la probabilidad de la enfermedad. Estudios desarrollados con gemelos, hermanos no gemelos y adoptados han permitido estimar que el componente genético contribuye desde un 40% a un 70% a la variación interindividual de la obesidad común (Elks *et al*. 2012). Así mismo, se ha observado que la influencia genética aumenta desde la infancia temprana hasta la adolescencia (Dubois *et al*. 2012). Gracias a los estudios de escaneo de genoma completo y de genes candidatos se ha demostrado la existencia de *loci* genéticos asociados con la forma más común de la obesidad (revisado por Rankinen *et al*. 2006 y Xia & Grant 2013).

La obesidad se caracteriza por una acumulación excesiva de tejido graso, que va acompañada por inflamación de bajo grado, alteración de la secreción de adipokinas, hipoxia y estrés oxidativo (Fernández-Sánchez *et al*. 2011). El estrés oxidativo se define como el desequilibrio entre la producción y degradación de especies reactivas de oxígeno (*reactive oxygen species* ROS) en el organismo. Las posibles causas de la relación entre la obesidad y un mayor estrés oxidativo pueden ser la hiperglucemia, un mayor nivel de ácidos grasos libres circulantes, una menor defensa antioxidante y la inflamación crónica asociada a la obesidad (Bondia-Pons *et al*. 2012, Furukawa *et al*. 2004).

El sistema de defensa antioxidante mantiene la homeostasis de ROS en el organismo. Comprende antioxidantes endógenos y exógenos. Los antioxidantes endógenos son fundamentalmente enzimas como glutatión peroxidasas (GPXs), catalasa (CAT), paraoxonasas (PONs), superóxido dismutasas (SODs) y peroxiredoxinas (PRDXs). Estas enzimas degradan las ROS a distintos niveles y compartimentos dentro y fuera de las células.

La presencia de polimorfismos de un solo nucleótido (*single nucleotide polymorphisms* SNPs) puede afectar al funcionamiento de las enzimas antioxidantes y de ese modo incrementar el riesgo de algunas enfermedades como el cáncer (Da Costa *et al*. 2012). Sin embargo, el efecto de las variaciones genéticas en genes relacionados con el estrés oxidativo no se ha estudiado en profundidad

en el contexto de la obesidad. Estudios que investiguen estos aspectos son necesarios para poder definir los mecanismos implicados en el desarrollo de las comorbilidades de la obesidad como el síndrome metabólico y la resistencia a la insulina.

Fundamento y objetivos

El grupo de investigación CTS-461 "Bioquímica de la Nutrición. Implicaciones Terapéuticas" centra una de sus líneas de investigación en el estudio de la obesidad infantil mediante distintos métodos que incluyen el estudio de biomarcadores de obesidad novedosos, marcadores de estrés oxidativo y análisis de variaciones genéticas mediante estudios de asociación de genes candidatos. En un primer lugar, el estudio de la asociación de variantes genéticas con la obesidad se realizó en genes previamente relacionados con la obesidad como el 11β-hidroxiesteroide deshidrogenasa tipo 1 (*HSD11B1*), neuropéptido Y (*NPY*), el gen asociado a obesidad y masa grasa (del inglés *fat mass and obesity associated, FTO*), entre otros. Estos genes se seleccionaron a partir de aquellos que se observaron sobreexpresados en el tejido adiposo visceral de niños prepúberes en un estudio llevado a cabo por nuestro grupo, además de los genes relacionados con masa grasa que se encontraban descritos en la bibliografía. A partir de este estudio se han publicado dos artículos que describen la asociación del SNP rs3753519 del gen *HSD11B1* (Appendix, I) y de las variantes rs16147 y rs16131 del gen *NPY* (Appendix, II) con un mayor riesgo de obesidad en niños.

El objetivo de la presente tesis fue identificar las variaciones genéticas localizadas en genes del sistema de defensa antioxidante que afectaran al riesgo de obesidad en niños prepúberes españoles, así como estudiar su impacto sobre el estrés oxidativo y las complicaciones metabólicas asociadas a la obesidad como son la resistencia a la insulina, la inflamación y el daño endotelial.

Metodología

Se trata de un estudio caso-control, transversal y multicéntrico que incluyó un total de 193 niños obesos (104 niños, 89 niñas) y 191 niños normopeso (110 niños, 81 niñas) reclutados en los centros de atención primaria de tres hospitales españoles: Hospital Universitario Reina Sofía y Valle de los Pedroches, ambos de Córdoba, y el Hospital Clínico Universitario de Santiago de Compostela. El protocolo se realizó de acuerdo con la Declaración de Helsinki y fue aprobado por el comité de ética de cada uno de los centros participantes.

A cada niño se le realizaron las medidas antropométricas y se le tomó la tensión arterial y una muestra de sangre tras ayuno nocturno. El análisis bioquímico general se realizó en cada uno de los hospitales participantes. El análisis de adipoquinas y biomarcadores de inflamación y daño endotelial se realizó en el equipo Luminex 200 mediante la tecnología XMap. Así mismo, se determinaron mediante ELISA las concentraciones de LDL oxidada (LDLox) y de proteína de unión a ácidos grasos 4 (FAPB-4), la proteína C reactiva (hsCRP) se determinó mediante un análisis turbidimétrico. La concentración de vitaminas en plasma (retinol, α-tocoferol y β-caroteno) se analizó mediante cromatografía líquida de alta resolución.

El sistema de defensa antioxidante se evaluó mediante la determinación de la capacidad antioxidante total del plasma (TAC), la actividad de las enzimas antioxidantes en eritrocitos [CAT, SOD, glutatión reductasa (GR) y GPX] y la actividad paraoxonasa 1 (PON1) en suero mediante distintos sustratos (paraoxon, fenilacetato, dihidrocumarina y diazoxón).

Mediante el uso de la base de datos dbSNP del Centro Nacional de Información Biotecnológica (NCBI) se elaboró una lista de 492 SNPs localizados en 47 genes candidatos elegidos previamente. Los tag SNPs se eligieron utilizando la base de datos HapMap. El genotipado fue realizado mediante la tecnología GoldenGate de Illummina. Tras la obtención de los primeros resultados, se siguieron una serie de pasos para el control de calidad de los resultados. Posteriormente se realizó un análisis de regresión logística ajustado por sexo y edad para el estudio de la asociación de cada SNP con el riesgo de obesidad infantil. Así mismo, se realizó un análisis de regresión lineal ajustado por sexo y edad para observar el efecto de cada SNP sobre los fenotipos estudiados. Adicionalmente, se ajustó este análisis por índice de masa corporal (IMC) con el fin de caracterizar mejor las asociaciones observadas. Para estos análisis el software utilizado fue PLINK 1.07.

Resultados

Análisis de antropometría y biomarcadores

En el grupo de los niños obesos los valores del IMC, IMC *z-score* y la circunferencia de cintura (CC) fueron mayores que en niños normopeso. Las tensiones arteriales sistólica (TAS) y diastólica (TAD) también mostraron un aumento con la obesidad. Se observó que los niveles de insulina en ayunas, así como el valor del índice de resistencia a insulina estaban incrementados en niños obesos, mientras que el ínice de sensibilidad a la insulina era menor en niños

obesos que en los normopeso. Los niveles de glucemia no mostraron diferencias. En cuanto al perfil lipídico, los niños obesos tenían menor concentración de colesterol total, colesterol HDL (lipoproteínas de alta densidad) y apolipoproteína A-I, y mayor concentración de triglicéridos, apolipoproteína B y FABP-4 que los niños normopeso. Las concentraciones de colesterol LDL (lipoproteínas de baja densidad) no mostraron diferencias entre grupos. Los niveles de resistina y leptina fueron mayores en niños obesos. Así mismo, las concentraciones de mieloperoxidasa, del inhibidor del activador del plasminógeno 1 activo y del total estaban aumentadas en los niños obesos, a diferencia de la concentración de metalopeptidasa 9, que no mostró diferencias significativas. Las interleukinas 6 y 8, la hsCRP y el factor de necrosis tumoral alfa estaban aumentados en el grupo de los niños obesos. La concentración de la proteína quimiotáctica de monocitos 1 no mostró diferencias entre grupos. La concentración de las transaminasas hepáticas alanina aminotransferasa y gamma-glutamil transpeptidasa fue mayor en niños obesos, y la de aspartato transaminasa fue menor en niños obesos frente a normopesos.

En cuanto al estado del estrés oxidativo, las LDLox no mostraron diferencias, mientras que la TAC fue significativamente menor en niños obesos. La actividad CAT se observó disminuída en eritrocitos de niños obesos, y no se observaron diferencias significativas en las actividades GR, GPX, SOD o PON1 entre grupos. Los niveles de las vitaminas α-tocoferol y β-caroteno fueron significativamente menores en niños obesos, y no se observaron diferencias en la concentración de retinol.

Genotipado y análisis de asociación

El estudio de asociación mostró que 19 SNPs del total de 492 analizados estaban asociados a la obesidad infantil. Un total de 7 SNPs incrementaban el riesgo de obesidad infantil, mientras que 12 SNPs conferían protección contra ella. Los SNPs están localizados en los siguientes genes: paraoxonasa 1 (*PON1*), catalasa (*CAT*), glutatión peroxidasas 4 (*GPX4*), 5 (*GPX5*) y 6 (*GPX6*), aldehído oxidasa 1 (*AOX1*), peroxiredoxina 5 (*PRDX5*), resistencia a la oxidación 1 (*OXR1*), respuesta al estrés oxidativo 1 (*OXSR1*) y sirtuína 2 (*SIRT2*).

Los resultados principales que se observaron en el estudio de asociación entre los SNPs protectores frente a la obesidad y los fenotipos estudiados fueron los siguientes:

- La variante rs854566 del gen *PON1* se asoció a un menor riesgo de obesidad, a mayor actividad lactonasa, arilesterasa y diazoxonasa, y a menor actividad

paraoxonasa de PON1 sérica en niños prepúberes (Chapter 3; Rupérez *et al*. 2013-a).

- Los SNPs rs475042 y rs494024 del gen *CAT* se asociaron a menor riesgo de obesidad infantil y a menor peso.
- Los SNPs rs757228, rs3746165 y rs8102188 del gen *GPX4* se asociaron a menor riesgo de obesidad y a menores niveles de marcadores de estrés oxidativo.
- Las variantes rs3731722 y rs1050887 del gen *AOX1* se asociaron a menor riesgo de obesidad y a menores valores leptina en niños prepúberes.
- El SNP rs4930698 del gen *PRDX5* se asoció a menor riesgo de obesidad y a menores valores de biomarcadores de inflamación y daño endotelial en niños prepúberes.
- El SNP rs1681887 del gen *OXR1* se asoció a menor riesgo de obesidad y a menores valores de marcadores de resistencia a la insulina y estrés oxidativo en niños prepúberes.
- El polimorfismo rs4955408 del gen *OXSR1* se asoció negativamente a la obesidad infantil y sus fenotipos, así como a menor resistencia a la insulina, daño endotelial y estrés oxidativo.

El estudio de la asociación entre los SNPs asociados a mayor riesgo de obesidad y los fenotipos estudiados generó los siguientes resultados principales:

- Las variantes en el promotor del gen *CAT* rs769214, rs8693316 y rs1049982 se asociaron a un mayor riesgo de obesidad y a mayores valores de sus fenotipos (Chapter 4; Rupérez *et al*. 2013).
- Las variantes rs28382586 y rs445870 del gen *GPX5* y rs406113 y rs11757000 del gen *GPX6* se asociaron a un mayor riesgo de obesidad en niños prepúberes.

Discusión

El aumento en la prevalencia de obesidad y sus alteraciones metabólicas asociadas como la diabetes de tipo 2 junto a la evidencia de estrés oxidativo asociado a la obesidad hacen necesario el estudio en profundidad de los mecanismos moleculares que conectan el estrés oxidativo con la obesidad.

Dada la ausencia de más información en este campo, nos dispusimos a llevar a cabo el estudio descrito en la presente tesis doctoral. Para tal fin, realizamos un estudio caso-control en un grupo de niños prepúberes españoles obesos y normopeso en los que estudiamos la asociación entre la presencia de SNPs en

genes del sistema de defensa antioxidante y el estado de obesidad y los valores de un gran número de marcadores metabólicos implicados en la resistencia a la insulina, el estrés oxidativo, la inflamación y el daño endotelial.

El análisis de los fenotipos observados mostró los resultados esperados en cuanto a marcadores relacionados con la obesidad como son el peso, el IMC, la CC, o las TAS y TAD, que se observaron aumentados en niños obesos prepúberes. Los biomarcadores de resistencia a la insulina, de metabolismo lipídico, de inflamación y de daño endotelial como el MPO (Olza *et al*. 2012-b) también se vieron alterados con la obesidad en estos niños, contribuyendo a la presencia de síndrome metabólico (Olza *et al*. 2011). Un descubrimiento novedoso fue que los niños obesos mostraron un sistema de defensa antioxidante debilitado, indicado por menores valores de actividad CAT, de TAC, de β-caroteno y de α-tocoferol (Rupérez *et al*. 2013-b). Estos datos concuerdan con los de otro estudio en el que se vieron mayores concentraciones de marcadores de estrés oxidativo como el malondialdehído y los grupos carbonilo, así como de actividad GPX, y menor concentración de glutatión reducido (GSH) en niños obesos. Sin embargo, en el mismo estudio no se encontraron diferencias en los valores de β-caroteno y de α-tocoferol, posiblemente debido a que el estudio incluía un menor número de sujetos (Codoñer-Franch *et al*. 2010). En nuestro caso, no encontramos diferencias en las actividades de PON1, GR, GPX o SOD, ni en el marcador de estrés oxidativo LDLox. La edad temprana de nuestra población en estudio podría ser una de las causas de la ausencia de mayores alteraciones en el estado de estrés oxidativo. No obstante, los cambios mencionados en el estado de defensa antioxidante podrían ser el origen del incremento de estrés oxidativo observado en la obesidad y sus complicaciones metabólicas.

El análisis de SNPs que puedan afectar al funcionamiento de enzimas antioxidantes, y por tanto al riesgo de padecer enfermedades como la obesidad, es un posible abordaje para aclarar los mecanismos implicados en las comorbilidades de la obesidad. Aunque se han llevado a cabo muchos estudios para caracterizar la asociación entre SNPs y obesidad infantil, la mayoría se han concentrado en SNPs de genes relacionados con la obesidad (Yu *et al*. 2012; Wheeler *et al*. 2013) y muy pocos han estudiado SNPs de genes antioxidantes. Algunos de los resultados descritos incluyen los SNPs rs662 (Q192R) y rs854560 (L55M) del gen *PON1*, que no mostraron asociación con obesidad en adolescentes (Baráth *et al*. 2006). En otro estudio los autores encontraron el SNP Ala16Val del gen *SOD2* asociado a la obesidad infantil en niños con hígado graso no alcohólico (El-Koofy *et al*. 2011). En nuestro estudio, analizamos 492 SNPs de genes del sistema de defensa antioxidante, de los cuales 12 resultaron ser protectores frente a la obesidad y 7 incrementaron el riesgo de obesidad en niños prepúberes. Ninguno de los SNPs descritos se había publicado previamente en lo que se refiere a su asociación con obesidad.

Uno de los principales descubrimientos de la presente tesis es el carácter protector de la variante génica rs854566 del gen PON1 en relación con la obesidad (Rupérez *et al*. 2013-a). Este SNP también se asoció a las actividades séricas de PON1 determinadas, diazoxonasa, lactonasa y arilesterasa. A pesar de estos resultados, las actividades PON1 no mostraron diferencias entre los grupos obeso y normopeso. Aún así, demostramos que la actividad lactonasa es un indicador fiable de la función de PON1, e insistimos en que debería ser utilizada en futuros estudios que tengan el objetivo de investigar el papel de PON1 en el desarrollo de la obesidad y la enfermedad cardiovascular.

Del mismo modo, describimos cómo el SNP rs769214 (-844A/G) del promotor del gen *CAT* estaba asociado con la obesidad infantil (Rupérez *et al*. 2013-b). Aunque esta variante génica no se asoció a la actividad CAT de eritrocitos, sí resultó estar asociada al FABP-4 en plasma, lo que podría indicar un posible papel de CAT en la disfunción del tejido adiposo presente en la obesidad.

También se encontraron un grupo de SNPs localizados en los genes *GPX4*, *GPX5* y *GPX6*. En el gen *GPX4*, los SNPs rs757228, rs3746165 y rs8103188 de la región 5' se asociaron con menor riesgo de obesidad. Aunque estas variantes se asociaron con marcadores de obesidad y a mayor TAC, ninguno se asoció con la actividad GPX de eritrocitos. Del mismo modo, en un estudio reciente, el SNP rs757228 tampoco se asoció a actividad GPX (Zanon-Moreno *et al*. 2013). Dos SNPs del gen *GPX5* (rs28382586 y rs445870) mostraron estar asociados positivamente con la obesidad y sus fenotipos relacionados. En cuanto al gen GPX6, dos SNPs (rs406113 y rs11757000) mostraron asociación con mayor riesgo de obesidad infantil. Hacen falta más estudios para poder entender el papel de estas variantes en la actividad enzimática.

En el gen aldehído oxidasa 1 (*AOX1*) se encontraron dos SNPs (rs3731722 and rs1050887) asociados a menor obesidad infantil así como a menor IMC, CC, peso y leptina. En un estudio reciente, Hartmann *et al*. describieron cómo el SNP rs3731722 (H1297R) provocaba un incremento en la eficacia metabólica de AOX1 (Hartmann *et al*. 2012). Teniendo en cuenta ambos hechos, una posible hipótesis para un estudio posterior sería que una actividad detoxificante aumentada de AOX1 podría tener un efecto sobre el metabolismo y un papel en la protección frente a la obesidad.

De un modo similar, encontramos los SNPs rs4930698 (*PRDX5*), rs1681887 (*OXR1*), rs4955408 (*OXSR1*) y rs892034 (promotor *SIRT2*) asociados negativamente con la obesidad. Se sabe que SIRT2 tiene un efecto inhibidor sobre la diferenciación adipogénica (Jing *et al*. 2007). Por tanto, se podría hipotetizar que la presencia del SNP rs892034 en el promotor de *SIRT2* podría incrementar su expresión en el tejido adiposo y así inhibir la diferenciación adipogénica,

proporcionando una posible explicación de su función protectora frente a la obesidad.

Estos resultados deben ser validados en estudios con un mayor número de individuos, y en distintas poblaciones para asegurar la calidad de los descubrimientos. La realización de estudios *in vivo* e *in vitro* que investiguen la función de estas variantes génicas en el tejido adiposo será clave para elucidar el papel de defensa antioxidante de estas enzimas en la protección frente al desarrollo de la obesidad y sus comorbilidades.

Conclusión

Los polimorfismos descritos localizados en genes del sistema de defensa antioxidante ejercen un efecto sobre el riesgo de obesidad en niños prepúberes españoles. Además, la presencia de estos SNPs influye sobre un gran número de fenotipos asociados con la obesidad, la resistencia a la insulina, la inflamación, el daño endotelial y el estrés oxidativo. Estos datos apoyan el papel del estrés oxidativo en la patogénesis de la obesidad y sus complicaciones metabólicas.

Limitaciones del estudio

A pesar del tamaño considerable de nuestra población objeto de estudio, un mayor número de participantes habría incrementado la significación de los resultados. No hemos validado los resultados en otras poblaciones. La mayoría de los SNPs observados no tienen un papel claro en la regulación genética o en la proteína codificada, indicando que son probablemente SNPs marcadores y no SNPs causantes de la enfermedad. Las asociaciones observadas perdieron su significancia tras el ajuste por comparaciones múltiples de Bonferroni.

References/Bibliografía

Bondia-Pons I, Ryan L and Martinez JA. Oxidative stress and inflammation interactions in human obesity. J Physiol Biochem 2012; 68: 701-711.

Da Costa, L.A., Badawi, A. and El-Sohemy, A. (2012) Nutrigenetics and modulation of oxidative stress. Ann Nutr Metab 2012; 60: 27-36.

Dubois L, Ohm Kyvik K, Girard M, Tatone-Tokuda F, Pérusse D, Hjelmborg J, Skytthe A, Rasmussen F, Wright MJ, Lichtenstein P, Martin NG. Genetic and environmental contributions to weight, height, and BMI from birth to 19 years of age: an international study of over 12,000 twin pairs. PLoS One 2012; 7: e30153.

Elks CE, Loos RJ, Hardy R, Wills AK, Wong A, Wareham NJ, Kuh D, Ong KK. Adult obesity susceptibility variants are associated with greater childhood weight gain and a faster tempo of growth: the 1946 British Birth Cohort Study. Am J Clin Nutr 2012; 95: 1150-1156.

Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González A, Esquivel-Chirino C, Durante-Montiel I, Sánchez-Rivera G, Valadez-Vega C, Morales-González JA. Inflammation, Oxidative Stress, and Obesity. Int J Mol Sci 2011; 12: 3117-3132.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M and Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004; 114: 1752-1761.

Gupta N, Goel K, Shah P, Misra A. Childhood obesity in developing countries. Epidemiology, determinants, and prevention. Endocr Rev 2012; 33: 48-70.

Han JC, Lawlor DA, Kimm SY. Childhood obesity. Lancet 2010; 375: 1737-48.

Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, Pérusse L, Bouchard C. The human obesity gene map: the 2005 update. Obesity (Silver Spring) 2006; 14: 529-644.

Xia Q, Grant SF. The genetics of human obesity. Ann N Y Acad Sci 2013; 1281: 178-190.

INTRODUCTION

INTRODUCTION

Childhood Obesity

Obesity is defined as an excessive accumulation of fat that causes a risk for health. In adults, a body mass index (BMI) higher than 25 kg/m^2 indicates overweight, and a BMI higher than 30 kg/m^2 indicates obesity. However, in children, the criteria used to classify individuals as overweight and obese need to take into account variations due to growth during normal development (Rolland-Cachera 2011). The most accepted classification is from Cole *et al*. (Cole *et al*. 2000) who established reference tables indicating the equivalence between the 25 and 30 kg/m^2 cut-offs from adults and children of both genders (Figure 1).

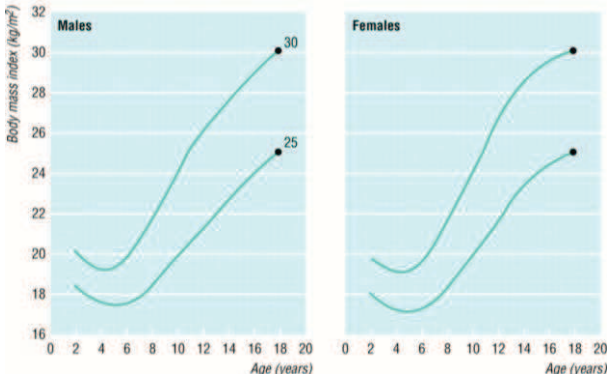

Figure 1. *International cut off points for body mass index by sex for overweight and obesity, passing through BMI 25 and 30 kg/m^2 at age 18 (data from Brazil, Britain, Hong Kong, Netherlands, Singapore, and United States) (Cole et al. 2000).*

According to the World Health Organization (WHO) childhood obesity is nowadays a global problem affecting a high number of low and middle-income countries, most of all in urban environments. In 2010, the prevalence of childhood overweight was higher than 42 million, of which 35 million belonged to developing countries (Gupta *et al*. 2012). Childhood obesity is currently a burden for healthcare systems, with its rates increasing dramatically worldwide (Ebbeling *et al*. 2002; Han et al. 2010) (Figure 2). In Spain, the ALADINO study was conducted in 2011 in a cohort of 7659 children from 6 to 10 years, and it revealed a prevalence of 26.1% for overweight and of 19.1% for obesity (Pérez-Farinós *et al*. 2013).

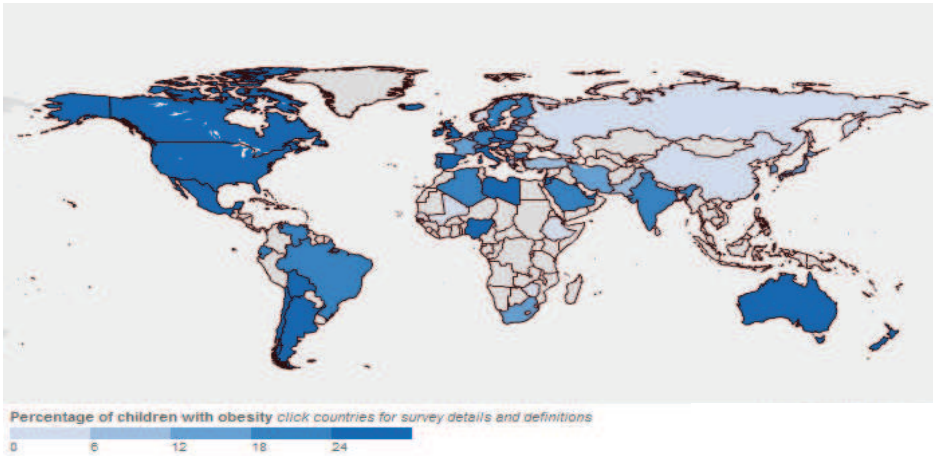

***Figure 2**. World map of childhood obesity prevalence. Data from the International Association for the Study of Obesity (IASO).*[1]

Obesity is a complex trait that stems from a complicated network of contributory components, including genomic and environmental factors, the aggregations of which increase the probability of disease. The main environmental cause of obesity is an imbalance between consumed and expended calories. In general, the present population has tended to consume an excessive amount of high caloric food. And with the same trend, people have evolved to a sedentary lifestyle with low physical activity at work, transport and social everyday life. However, lifestyle fails to explain by itself the whole etiology of obesity and genetics seem to play an important role in the etiology of obesity.

Metabolic syndrome in children

Metabolic syndrome is defined as the cluster of anthropometric, physiological and biochemical alterations that increase the risk of an individual to develop type 2 diabetes and cardiovascular diseases (Nelson & Bremer 2010). High blood pressure, hipertriglyceridemia, low high-density lipoprotein (HDL)-cholesterol and an altered glucose metabolism characterize this syndrome. Insulin resistance is one of the key players together with adiposity, especially visceral fat (Figure 3).

[1] http://www.iaso.org/resources/world-map-obesity/?map=children

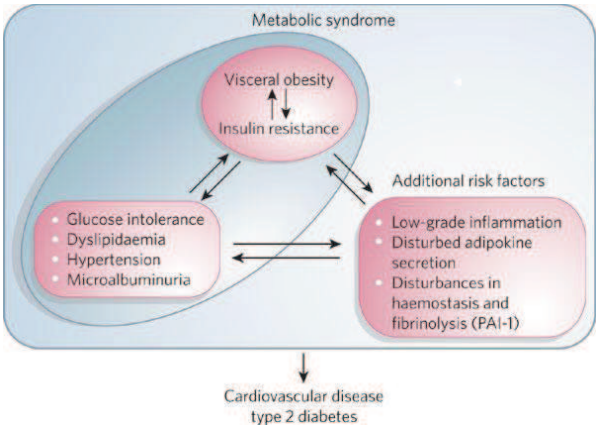

Figure 3. *Classical features and new components of metabolic syndrome and its core features insulin resistance and visceral obesity (Van Gaal et al. 2006). PAI-1: plasminogen activator inhibitor-1.*

However, in children there is currently a vigorous debate on the diagnosis and phenotypic expression of metabolic syndrome, especially before puberty. Single cut-off points cannot be used to define body composition or metabolic abnormalities in children, because these change with age, sex, and pubertal stage. To this date, published criteria for childhood metabolic syndrome differ in the parameter chosen to evaluate excess adiposity. Some authors used BMI (Weiss *et al.* 2004; Viner *et al.* 2005), while others selected waist circumference (WC) (Cook *et al.* 2003; De Ferranti *et al.* 2004; Cruz *et al.* 2004) because of its strong correlation with visceral adipose tissue. Also, there appeared to be no well-established reference standards for plasma lipids. Due to these differences, the frequencies of metabolic syndrome in children vary according to the criteria that are used.

Despite of these issues, it is clear that obese children have an increased chance of being obese in their adulthood and a higher risk of developing metabolic syndrome, diabetes and cardiovascular disease at early ages. Indeed, oxidative stress and metabolic syndrome alterations appear already in childhood, especially in obese children (Codoñer-Franch *et al*. 2011; Ford *et al*. 2008; Rupérez *et al.* 2013-a,b; Olza *et al.* 2011). For this reason, both prevention and treatment of childhood obesity and its derived metabolic complications are key aspects where we should take part in order to increase the quality of life of these children as well as to avoid an overload of healthcare systems in the future.

Oxidative stress in obesity

Oxidative stress is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism. The link between obesity and enhanced oxidative stress might be due to the hyperglycaemia, high circulating free fatty acids (FFA), decreased antioxidant defenses and chronic inflammation associated with obesity (Bondia-Pons 2012, Furukawa 2004). The antioxidant defense system (ADS) maintains ROS homeostasis in the cells (Figure 4). It comprises both endogenous and exogenous antioxidants. Endogenous antioxidants primarily include enzymes such as glutathione peroxidases (GPXs), glutathione reductase (GR), catalase (CAT), paraoxonases (PONs), superoxide dismutases (SODs) and peroxiredoxins (PRDXs). These enzymes degrade ROS at different levels and in different compartments inside and outside of the cells.

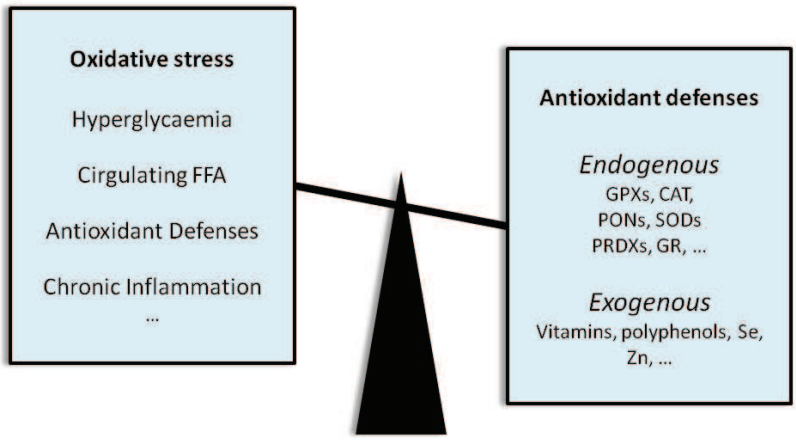

Figure 4. *Oxidative stress producing and scavenging systems. CAT: catalase; FFA: free fatty acids; GPXs: glutathione peroxidases; GR: glutathione reductase; PONs: paraoxonases; PRDXs: peroxiredoxins; SODs: superoxide dismutases.*

Obesity is characterized by an excessive accumulation of fat in adipose tissue, which is known to be accompanied by low-grade inflammation, adipokine secretion disregulation, hypoxia and **oxidative stress** that affect peripheral tissues (Fernández-Sánchez *et al.* 2011; Bondia-Pons *et al.* 2012; Ouchi *et al.* 2011).

Oxidative stress is the imbalance between the production of reactive oxygen species and the capacity to remove them or repair the provoked damage.

It is hypothesized that oxidative stress is one of the links between fat accumulation-derived alterations and the appearance of a cluster of health problems including adipokine secretion alteration, inflammation and **insulin resistance**, (Figure 5) (Furukawa *et al.* 2004; Houstis *et al.* 2006; Hotamisligil 2006).

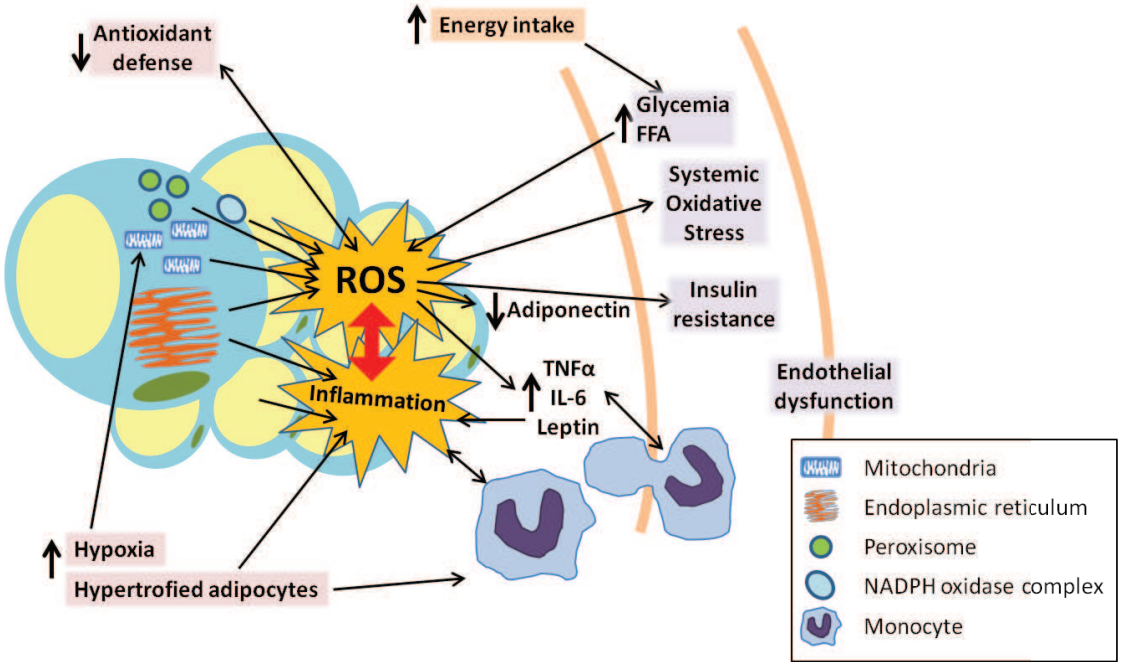

Figure 5. *Scheme representing the origin of ROS and inflammation in the context of obesity. Obesity is associated a high energy intake, which increases glycemia and circulating FFA. These increase ROS in the cells due to the over-activation of mitochondrial electron transport chain and the endoplasmic reticulum. The obesity associated fat accumulation leads to inflammation, hypertrophied adipocytes and hypoxia. Moreover, oxidative stress worsens inflammation and alters adipokine secretion. Many of these phenomens activate the monocyte infiltration in adipose tissue that worsens the inflammatory proccesses. Hypoxia also increases glucose uptake, and thus mitochondrial function, further contributing to ROS production. Through these mechanisms, ROS contribute to the development of insulin resistance, systemic oxidative stress and endothelial damage. FFA: free fatty-acids; IL-6: interleukin 6; NADPH: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; TNFα: tumor necrosis factor alpha.*

Insulin resistance is the condition in which cells fail to respond to the normal actions of insulin, impairing its proper use and leading to hyperglycemia and hyperinsulinemia.

Genetics of obesity

As already mentioned before, genetics play an important role in the etiology of obesity. There are certain genetic factors that provoke inter-individual differences in obesity susceptibility, even in the same environment and lifestyle (Lyon & Hirshhorn 2005). Thus, studies investigating the impact of these genetic differences on disease susceptibility are essential. Studies in twins, no-twin siblings, and adoptees have estimated that genetic components contribute from 40% to 70% to the inter-individual variation in common obesity (Elks *et al*. 2012). It should also be emphasized that heritability estimates have been shown to increase from early childhood through adolescence (Dubois *et al*. 2012). The presence of monogenic or endogenous obesity in which a specific gene such as melanocortin receptor (*MC4R*), propiomelanocortin (*POMC*), leptin receptor (*LEPR*) or leptin (*LEP*) harbors mutations that interfere with the synthesis of the resulting functional protein (Hinney *et al*. 2010) is also well known, although less frequent. In addition, it is also hypothesized that transgenerational changes may occur in obesity through epigenetic mechanisms; meaning that the mother's weight gain during pregnancy could predispose the children to overweight and obesity.

However, the common obesity is considered a polygenic disease, and the presence of a high number of small variations such as **single nucleotide polymorphisms** (SNPs) spread across the genome in many genes is now considered one of the main genetic components in the ethiology of obesity. More than 400 positive associations have been observed between genetic variations and obesity phenotypes (Figure 6). A total of 32 loci with genome-wide significance have been found through genome-wide association studies (GWAS)(Vimaleswaran *et al.* 2012; Fall *et al.* 2012), and, interestingly, candidate gene association studies allowed for the discovery of 127 candidate genes (Wang *et al.* 2011; Rankinen *et al.* 2006; Xia & Grant 2013).

Single nucleotide polymorphism DNA sequence variation of a single nucleotide which differs from that of another individual or chromosome in the same position.

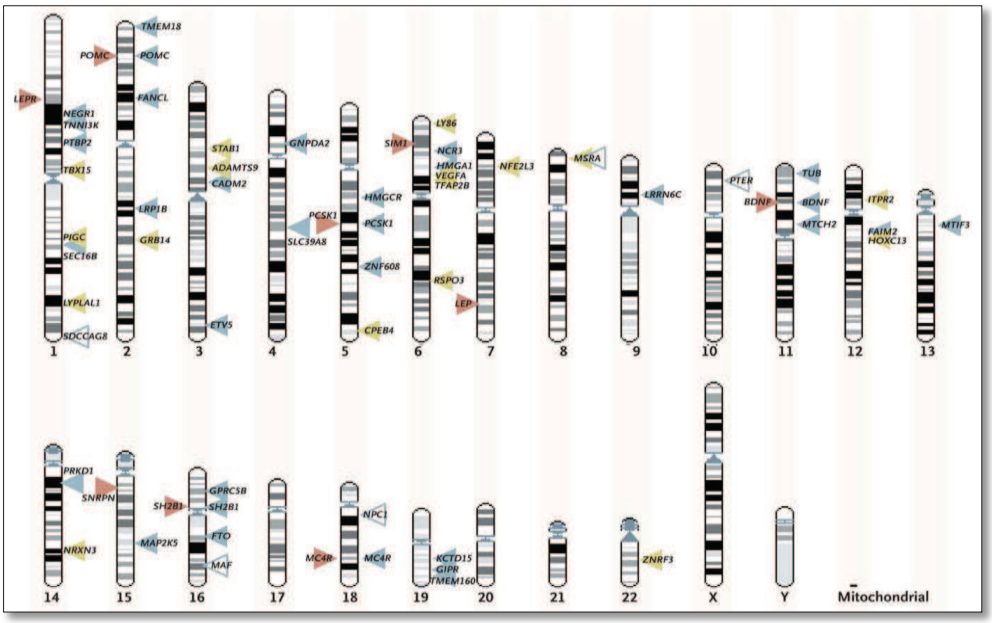

Figure 6. *Genome map of loci associated with obesity and its phenotypes (McCarthy 2010).*

Genetic studies

Linkage studies

These studies are carried out with families and related individuals and allow for the discovery of hot spots in the genome that show association to a certain disease, without previous knowledge of a candidate gene. They have a low resolution and are less efficient for finding genes affecting polygenic diseases such as obesity, but are a good first approach which helps find candidate regions in the genome.

Association studies

In these studies, the association between a disease and the presence of a genetic marker is investigated in a population of non-related individuals. This simplifies the recruitment process, although population stratification may occur. Studies can be prospective (cohorts) or retrospective (case-control).

In case-control studies, subjects are recruited according to the presence of a certain disease or phenotype. Whereas in cohort studies, individuals are recruited first, and observed during a certain time in which the apparition of the disease is checked for.

Once the subjects are recruited, the study of the association of SNPs with the disease starts (Figure 7). This step can be done through two different approaches: the candidate gene studies and GWAS.

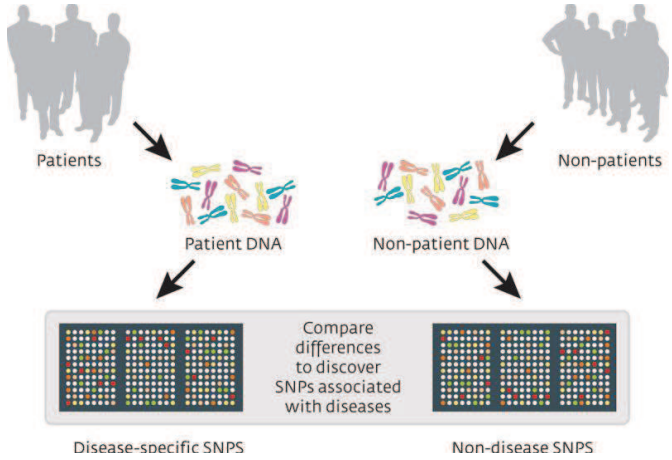

Figure 7. *Scientists compare DNA from thousands of patients to DNA from thousands of control subjects, looking for differences ('SNPs' or single nucleotide polymorphisms) between the two groups. © Pasieka, Science Photo Library.*

Candidate gene association studies

This was the selected approach for the present PhD thesis. In this type of studies, SNPs are chosen according to previous knowledge of biochemical pathways that relate a gene with a disease. In other words, a previous hypothesis is needed. These studies are highly valuable when the risk allele frequency or the effect sizes are low, because the power of the association is increased due to the previous election of the SNPs (as opposed as they being randomly spread across the genome). In addition, they are useful for validation studies in different populations.

Nevertheless, in order to obtain high quality reliable results, a series of steps in the study design must be followed. These include the selection of SNPs from genes of common pathways, **missense SNPs** and SNPs in **linkage disequilibrium** (LD), the known frequencies in different populations and sample size during the

Missense SNP is a SNP located in the coding region of a gene that affects the amino acid of the protein.
Linkage disequilibrium non-random association of alleles appreciated by the difference between the observed and the expected combination of the alleles based on their frequencies.

selection of the SNPs; and a exhaustive quality control and data analysis once the genotyping is completed (Jorgensen *et al*. 2009).

Genome-wide association studies (GWAS)

GWAS examine the association of hundreds of thousands of SNPs spread all along the genome. Large groups of individuals are needed in order to find significant associations of the SNPs with a disease (Manolio 2010). Since the first GWAS in 2005, more than 200 diseases have been studies with this method, finding more than 4000 associations.

These studies are based in the LD principle, in which common SNPs are inherited in blocks between generations, allowing for some of these SNPs to be used as **tag SNPs** (Stram 2004). It is necessary to mention that from all the studied SNPs only 12% are located in coding regions, 40% are intergenic and 40% intronic (Figure 8). These data suggest that introns and intergenic regions definitely play an important role in genetic regulation (Hardy 2009).

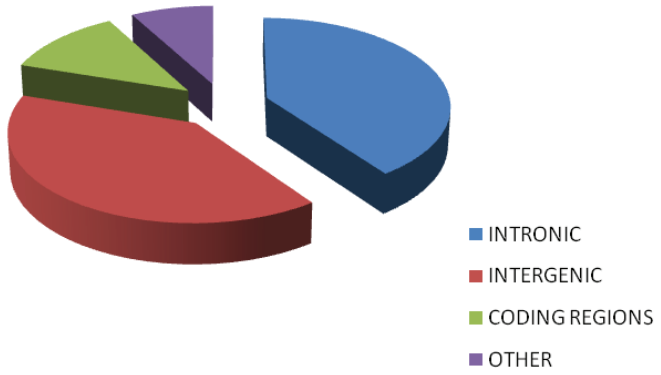

Figure 8. *Genomic location of SNPs described by GWAS.*

Despite their potential, GWAS have also a series of negative aspects to be considered. First of all, it is truly difficult to find the functional explanation of an association between a SNP and a disease, because most of the times SNPs are localized in unknown genes or genomic regions. Secondly, the found associations usually differ between populations. And third, GWAS do not include SNPs with a minor allele frequency (MAF) lower than 1%, thus missing a lot of genetic information that affects complex diseases as obesity.

AIMS OF THE STUDY

BACKGROUND AND JUSTIFICATION

The research group CTS-461 "Biochemistry of Nutrition. Therapeutical implications" focuses one of its lines of work on childhood obesity through different approaches. These include novel obesity biomarkers analysis, oxidative stress markers and genetic variation analysis through candidate gene association studies. For all of these tasks, a population of more than 1000 obese and normal-weight children have been recruited at the collaborating hospitals of "Reina Sofía" and "Valle de los Pedroches", both in Córdoba, the "Clinical University Hospital" in Santiago de Compostela and the "Clinical University Hospital Lozano Blesa" in Zaragoza.

The first approach in the study of genetic variants affecting childhood obesity risk was to observe SNPs from the most well known obesity-related genes. Arising from this project, a previous PhD thesis was defended in the group (Olza J. 2011). In addition, a series of articles were published and others are in progress. Those publications are included in this thesis as additional publications (Appendix), as this thesis focuses on ADS-related genes.

The main published findings regarding obesity-related SNPs, attached in the Appendix, are the following. The novel SNP rs3753519 from the 11β-hydroxysteroid dehydrogenase type 1 (*HSD11B1*) gene was found to be positively associated with childhood obesity [Odds ratio (OR) = 1.97, 95% Confidence Interval (CI) 1.23-3.16, p = 0.004] (Olza *et al*. 2012). Also, variants rs16147 and rs16131 in the neuropeptide Y (*NPY*) gene where associated with higher childhood obesity risk (OR = 1.38, 95%CI 1.08-1.78, p = 0.010; OR = 1.69, 95%CI 1.18-2.41, p = 0.004 respectively) (Olza *et al*. 2013). Previously known SNPs from the fat mass and obesity (*FTO*) gene were validated in their association with obesity in children, as well as with obesity biomarkers (Olza *et al.* submitted to BMC Medical Genetics in 2013).

Following this research, the group obtained a grant (PI-0296/2007) to study the potential implication of SNPs of ADS-related genes in the risk of obesity during childhood. The present PhD thesis arises from that project, which allowed for the identification of genetic variants that affect oxidative stress status and ultimately, obesity and its metabolic complications.

HYPOTHESIS

The starting hypothesis was that obese children show an altered ADS and higher cellular oxidative stress, all related with early onset of obesity and metabolic syndrome features in prepubertal obese children. In addition, we hypothesized that the oxidative stress could have an underlying genetic cause due to the presence of SNPs in ADS-related genes.

AIMS

The present study aimed to determine the frequency of 492 SNPs selected from 47 genes related with the ADS and free radical metabolism, as well as to study their association with obesity risk in a population of prepubertal Spanish children.

General aims

I. To review the current literature regarding the impact of SNPs for oxidative stress related genes on obesity risk.

II. To obtain blood samples for plasma and serum analysis, as well as for genomic DNA extraction.

III. To carry out a case-control association study in a cohort of 193 obese and 191 normal-weight healthy Spanish children.

IV. To select from 2 to 15 SNPs per gene among the 47 candidate ADS-related genes from the National Center for Biotechnology Information (NCBI) database dbSNP and from the HapMap Project database until 492 SNPs were reached.

V. To determine the frequency of the selected SNPs.

Specific aims

I. To study the observed differences of the analyzed biomarkers between the obese and normal-weight children.

II. To analyze the association of the presence of the selected SNPs with the risk of obesity.

III. To study the association between the selected SNPs and the main phenotypes and features related with obesity, metabolic syndrome, insulin resistance, oxidative stress, inflammation and endothelial damage.

Methodology

METHODOLOGY

Experimental design

This was a case-control, cross-sectional, multicentre study, in which a total of 384 prepubertal children aged between 3 and 13 were recruited from primary care centers at three Spanish hospitals (University Hospital "Reina Sofía" and Hospital "Valle de los Pedroches", both in Córdoba; and Clinical University Hospital of Santiago de Compostela). Pubertal stage was assessed following Tanner classification (Tanner 1962). The children were classified as obese or normal-weight following Cole's definition (Cole *et al*. 2000). A total of 193 children were grouped as obese (104 boys and 89 girls) and 191 (110 boys and 81 girls) as normal-weight healthy controls.

Inclusion criteria were: absence of disease related with nutritional status or endogenous obesity and prepubertal stage. Exclusion criteria were: the presence of disease or undernutrition and the use of medication that alters blood pressure (BP) or glucose or lipid metabolism and pubertal stage.

Following an initial assessment at school or a primary-care center, children who met the inclusion criteria were invited for a clinical examination at a participating hospital. The parents or guardians of the children were informed concerning the purpose and procedures of the study before written consent was obtained; all of the children agreed to participate in the study. Sex hormone levels were measured to confirm that the children were prepubertal.

The protocol was performed in accordance with the Declaration of Helsinki (Edinburgh 2000, revised), the recommendations of the Good Clinical Practice of the CEE (Document 111/3976/88, July, 1990) and the current Spanish regulations dictating clinical investigations in human subjects (RD 223/04 on Clinical Assays). The present study was approved by the Ethics Committee on Human Research of the University of Granada, the Ethics Committee of the Reina Sofía University Hospital of Córdoba and the Bioethics Committee of the University of Santiago de Compostela.

Anthropometry

After a detailed clinical history and an exhaustive physical examination, anthropometric measurements were performed by a single examiner with the children in bare feet and dressed in their underwear. Body weight (kg), height

(cm) and WC (cm) were measured using standardized procedures and were used to calculate the BMI of the children with the formula: weight (kg)/height (m^2). The BMI *z*-score was also calculated as the difference between the observed BMI value and the mean BMI of the reference population, divided by the standard deviation according to sex and age, the reference used was that from Sobradillo *et al*. (2004). Children were classified as obese or normal-weight according to BMI, using the age- and sex-specific cut-off points proposed by Cole *et al*. (Cole *et al*. 2000) (linked to adult cutoffs of 25 and 30 kg/m^2). In the same way, their prepubertal stage was assessed by Tanner classification (Tanner 1962). Systolic and diastolic BP was measured three times for each individual by the same examiner, according to international recommendations.

Biochemical measurements

Blood samples were drawn from the antecubital vein after the children had fasted overnight. A complete blood count and routine biochemical analyses were performed at the Hospital laboratory. Serum from one EDTA-coated tube was centrifuged 10 minutes at 4 °C and 1750 g and the plasma was frozen at -80 °C for future analysis of specific biomarkers. The buffy coat containing white blood cells was collected in a 1.5 ml tube and stored at -80 °C for DNA extraction.

In the general biochemical analysis the following parameters were measured: glucose, insulin, triacylclycerols (TG), HDL-cholesterol, low density lipoprotein (LDL)-cholesterol, total cholesterol, apolipoprotein A-I (ApoA-I), apolipoprotein B (ApoB), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamil transferase (GGT).

The homeostasis model assessment for insulin resistance index (HOMA-IR) (Matthews *et al*. 1985) was calculated using fasting insulin and glucose levels following the formula:

$$HOMA - IR = \frac{Glucose\,\frac{mmol}{l} \times Insulin\,\frac{\mu U}{ml}}{22.5}$$

The corresponding value of QUICKI (Quantitative Insulin Sensitivity Check Index) (Katz *et al*. 2000) was calculated according to the following formula:

$$QUICKI = \frac{1}{\log(Insulin\,\frac{mU}{l}) + \log(Glucose\,\frac{mg}{dl})}$$

Adipokines and biomarkers of inflammation and endothelial damage

Adipokines (adiponectin, resistin and leptin) and biomarkers of inflammation (IL-6, IL-8, TNF-α and MCP-1) and endothelial damage [Matrix metallopeptidase 9 (MMP-9), myeloperoxidase (MPO) and total and active plasminogen activator inhibitor 1 (PAI-1)] were measured by X-Map technology with LINCOplex kits (see list below) using the Luminex® 200™ (Luminex Corporation) according to the manufacturer's instructions.

- Human Serum Adipokine Panel A (HADK1-61K-A-03; LINCOplex Research): Adiponectin, resistin and active PAI-1.
- Human Cardiovascular Disease Panel 1 (HCVD1-67AK-06; LINCOplex Research): MMP-9, MPO and total PAI-1.
- Human Serum Adipokine Panel B (HADK2-61K-B-07; LINCOplex Research): IL-6, IL-8, MCP-1, leptin and TNF-α.

X-Map technology allows for the detection and analysis of up to 100 analytes per well of a 96-well plate. This technology combines fluidics, optics and digital signal processing. Color-coded tiny beads, called microspheres are each assigned a specific reagent for the analytes that we wish to determine. Once the reaction takes place between our sample and the reagents bound to the microsphere, the flow-cytometer detects the color of the microsphere and the signal due to the bound sample (Figure 9).

Oxidized LDL (Ox-LDL) particles were analyzed with an ELISA kit (BI-20042; Biomedica Medizinprodukte) according to the manufacturer's instructions. The inflammation marker high-sensitivity C-reactive protein (hsCRP) was determined with a turbidimetric immunoassay (Dade Behring).

Fatty acid-binding protein 4 (FABP-4) concentrations were measured using ELISA (RD191036200R; BioVendor). Retinol, α-tocopherol and β-carotene levels were analyzed by high-pressure liquid chromatography (HPLC), as described previously (García-Rodríguez *et al*. 2012).

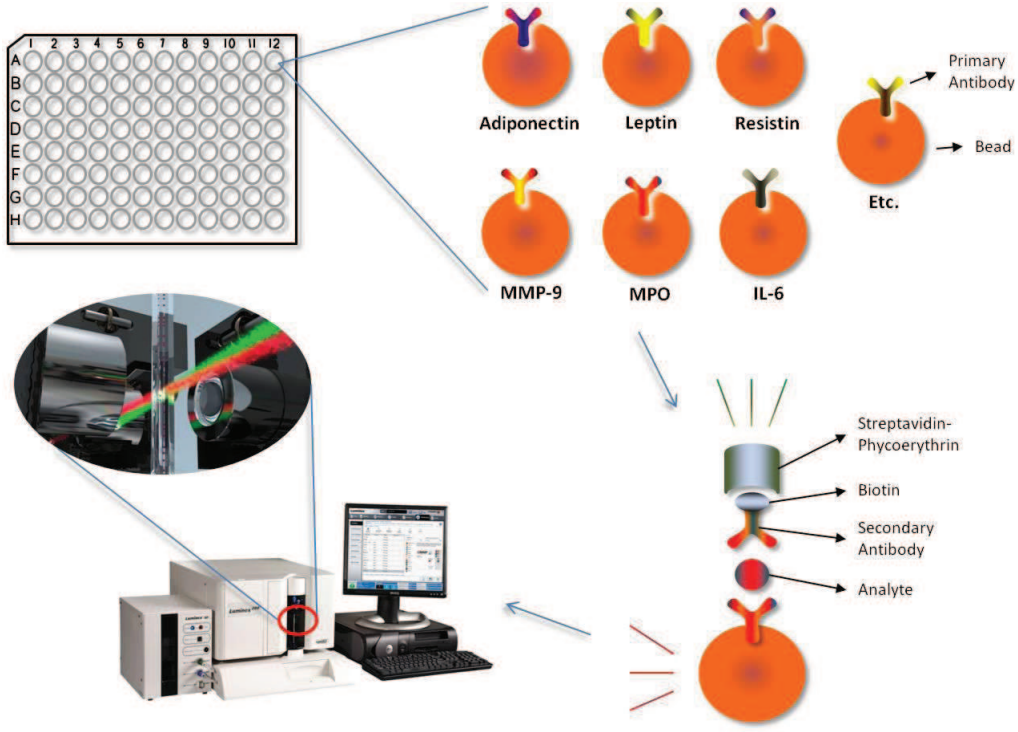

Figure 9. *XMap technology standard protocol for LINCOplex kits (scheme by Alcalá-Bejarano J). IL-6: interleukin 6; MMP-9: matrix metalloproteinase 9; MPO: myeloperoxidase; PAI-1: plasminogen activator inhibitor 1.*

Antioxidant defense system evaluation

Plasma total antioxidant capacity (TAC) was assessed using the spectrophotometric antioxidant assay kit (709001; Cayman).

Activity of the antioxidant enzymes in erythrocytes

Hemoglobin (Hb) concentration in the blood samples was determined spectrophotometrically by the colorimetric cyanmethemoglobin method (Drabkin 1948), using Sigma Diagnostic reagents.

Catalase activity

CAT activity in erythrocytes was assayed by the spectrophotometric method described by Aebi (Aebi 1984). After adjusting the Hb concentration to 1 g/l, 10 µL of simple were added to 90 µl of phosphate buffer 50 mM, pH 7.0, in a 96-well plate. Then, 50 µl of H_2O_2 30 mM were added to the reaction and the decrease in absorbance at 240 nm was monitored immediately during 40 s. CAT activity was expressed as K_{Hb} (kU/g Hb).

Superoxide dismutase activity

SOD activity was determined following the xantine/xantine-oxidase method for the generation of superoxide radicals, which oxidize cytrochrome c thus generating a color that can be measured at 450 nm. The depletion of superoxide by SOD is then measured to determine its activity (McCord *et al.*, 1969). After adjusting the Hb concentration to 1 g/l, the supernatant was extracted with chloroform-ethanol. Then, 40 µl of supernatant were mixed with 240 µl of reagent ferricytochrome c 50 µM, xantine 1 mM, $NaHCO_3$ 20 mM, pH 10.0, EDTA 0.1 M in a 96-well plate. The mixture was incubated during 90 s and then 20 µl of xantine oxidase 15 mU/ml were added. The absorbance was monitored at 450 nm during 3 min. Enzyme activity was expressed as U/mg Hb.

Glutathione reductase activity

GR activity was determined by measuring the oxidation rate of NADPH in the presence of oxidized glutathione (GSSG) (Carlberg *et al*. 1985). After adjusting the Hb concentration to 10 g/l, 30 µl of simple were added to 205 µl of GSSG 3.7 mM (G-4376; Sigma) in phosphate buffer 147 mM, EDTA 0.47 mM, pH 7.2, in a 96-well plate. The mixture was incubated during 5 min at 37 °C and then 40 µl NADPH 2.25 mM in $NaHCO_3$ were added to each of the wells. Absorbance at 340 nm was monitored during 5 min. GR activity was expressed as µmol/g Hb/min.

Glutathione peroxidase activity

Erythrocyte GPX activity was determined by the coupled enzyme spectrophotometric method with tert-butyl hydroperoxide (t-BOOH) as substrate (Flohé *et al*. 1984). After adjusting the Hb concentration to 10 g/l, 25 µl of sample were added to 125 µl of phosphate buffer 100 mM, 1 mM EDTA, pH 7.4, 0.1 M sodium azide, 25 µl GR enzyme (2.4 U/ml) (G-4751; Sigma) en in phosphate buffer 0.1 M, 25 µl GSH 10 mM (G-4251; Sigma) in phosphate buffer 0.1 M and 25 µl NADPH 1.25 mM (N-1630; Sigma) in $NaHCO_3$ buffer, in 96-well plates. The mixture was incubated during 3 min at 37 °C. Then 25 µl t-BOOH 12 mM were added to start the reaction. The decrease in absorbance at 340 nm was monitored during 5 min. GPX activity was expressed as mol/g Hb/min.

Determination of Paraoxonase 1 activities

Serum paraoxonase 1 (PON1) activity was determined by using four different substrates (paraoxon, phenylacetate, dihydrocoumarin and diazoxon) to obtain the best information on regards to this enzyme activity.

Paraoxonase activity was measured using 190 µl of paraoxon solution (1.0 mM) freshly prepared in 50 mM glycine buffer pH 10 containing 1 mM $CaCl_2$ with 10 µl of serum , incubating at 37 °C (Hernández *et al*. 2004). The liberation of p-nitrophenol upon enzymatic hydrolysis was measured spectrophotometrically at 405 nm. Hydrolysis of paraoxon was also determined in the presence of 1.0 M NaCl at pH 10 (referred to as salt-stimulated paraoxonase) as this method has been followed by some authors (data not shown in this thesis). Paraoxonase activity was expressed in U/l using p-nitrophenol molar extinction coefficient ε=16 l/mmol cm.

Arylesterase was measured using phenylacetate as the substrate. The assay mixture included 100 µl of a 1:50 dilution of serum, and a mixture of 1 ml of 8 mM phenylacetate in water plus 1 ml of 100 mM Tris–acetate buffer pH 7.4 containing 10 mM $CaCl_2$. Initial rates of hydrolysis were monitored spectrophotometrically following the formation of phenol at 270 nm at 25 °C (Junge *et al*. 1984). Arylesterase activity was expressed as kU/ml using phenol molar extinction coefficient ε=1,48 l/mmol cm.

Hydrolysis of dihydrocoumarin (an aromatic lactone, referred to as lactonase activity) was measured by the method described by Draganov et al. (Draganov *et al*. 2000) with minor changes. The assay medium contained 1 mM substrate (from a 100 mM stock solution, dissolved in methanol) in 50 mM Tris–HCl buffer pH 7.0

containing 1 mM $CaCl_2$, in a final volume of 1 ml. The reaction was initiated by the addition of 10 µl of serum and followed at 37 °C by monitoring the increase in UV absorbance at 270 nm. Lactonase activity was expressed in U/ml, using the molar extinction coefficient, ε=1.30 l/mmol cm.

Diazoxonase activity was determined by a continuous spectrophotometric assay. The assay contained 0.1 M Tris–HCl buffer pH 8.5, 2.0 M NaCl, 2.0 mM $CaCl_2$, and 500 µM diazoxon (Techno Spec) in a volume of 1 ml and the reaction was initiated by the addition of 5 µl of serum at 37 °C (Richter *et al*. 1999). The appearance of 2-isopropyl-4-methyl-6-hydroxy pyrimidine (IMHP) was continuously monitored at 270 nm. Rates of conversion of diazoxon to IMHP were calculated based on the molar extinction coefficient of IMHP ε=3.03 l/mmol cm and diazoxonase activity was expressed as U/l.

Arylesterase, lactonase and diazoxonase enzyme assays were performed in a Perkin-Elmer Lambda-2 spectrophotometer. PON1 paraoxonase and salt-stimulated paraoxonase activities were performed in a multidetection microplate reader (BIOT-TECH: Synergy HT). Samples were assayed in duplicate, and the average value was used for analysis. Non-enzymatic hydrolysis for each substrate was measured by using buffer, instead of serum, and was subtracted from the total rate of hydrolysis.

SNP selection and genotyping

SNP selection

A list of 47 candidate genes was elaborated after a literature review of genes related with the ADS (Table 1). From this list, SNPs located in their area of the genome were searched for according to the following criteria:

- Missense SNPs no matter their frequency in Caucasian population.
- Intronic SNPs with minor allele frequency (MAF) higher or equal to 0.05 in Caucasian population.
- SNPs in 3' and 5' region, including SNPs in the promoter region, with MAF higher or equal to 0.05 in Caucasian population.
- Tag SNPs were selected in order to optimize the process of genotyping, with MAF equal or higher to 0.05 in Caucasian population, with an r^2 equal or higher to 0.8. However, concerning previously known SNPs from important genes, we included them no matter their LD with the rest of SNPs from the same gene.

A total of 492 SNPs (Table of genotyped SNPs, Appendix) were selected. The selection of SNPs was done by using the National Center for Biotechnology Information database dbSNP[2]. Tag SNP selection was done with the HapMap database[3].

Table 1. Selected genes for the SNP search (alphabetic order).

Gene symbol	Gene name
ALB	albumin
AOX1	aldehyde oxidase 1
CAT	catalase
CCL5	chemokine (c-c motif) ligand 5
CCS	copper chaperone for superoxide dismutase
CRP	c-reactive protein
CYBA	cytochrome b-245, alpha polypeptide
CYGB	cytoglobin
DGKG	diacylglycerol kinase, gamma 90kDa
DHCR24	24-dehydrocholesterol reductase
DUOX1	dual oxidase 1

[2] http://www.ncbi.nlm.nih.gov/snp/
[3] http://www.hapmap.org/

Gene symbol	Gene name
DUOX2	dual oxidase 2
GPX1	glutathione peroxidase 1
GPX2	glutathione peroxidase 2 (gastrointestinal)
GPX3	glutathione peroxidase 3 (plasma)
GPX4	glutathione peroxidase 4
GPX5	glutathione peroxidase 5
GPX6	glutathione peroxidase 6 (olfactory)
GPX7	glutathione peroxidase 7
GSR	glutathione reductase
GSS	glutathione synthetase
GSTZ1	glutathione s-transferase zeta 1
LPO	lactoperoxidase
MGST3	microsomal glutathione s-transferase 3
MPO	myeloperoxidase
NCF2	neutrophil cytosolic factor 2
NOS2A	nitric oxide synthase 2, inducible
NOX5	NADPH oxidase, ef-hand calcium binding domain 5
OXR1	oxidation resistance 1
OXSR1	oxidative stress responsive 1
PON1	paraoxonase 1
PON3	paraoxonase 3
PRDX1	peroxiredoxin 1
PRDX2	peroxiredoxin 2
PRDX3	peroxiredoxin 3
PRDX4	peroxiredoxin 4
PRDX5	peroxiredoxin 5
PRDX6	peroxiredoxin 6
PTGS1	prostaglandin-endoperoxide synthase 1 (cyclooxygenase-1)
PTGS2	prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2b)
SCARA3	scavenger receptor class a, member 3
SEPP1	selenoprotein p
SIRT1	sirtuin 1
SIRT2	sirtuin 2
SOD1	superoxide dismutase 1, soluble
SOD2	superoxide dismutase 2, mitochondrial
SOD3	superoxide dismutase 3, extracellular

Sample genotyping

For the genotyping we used the Illumina GoldenGate technology in 96 sample arrays using 250 ng of total genomic DNA as a sample.

Sample preparation

Genomic DNA was extracted from buffy coats using QIAamp Blood kit (Qiagen). A series of random samples were run in an electrophoresis of agarose gel in order to check the integrity of the DNA.

Genotyping

Genotyping was performed using the GoldenGate protocol (Illumina) on 96-well format Sentrix® arrays. In this technology two allele-specific oligo (ASO) and one locus-specific oligo (LSO) are used for each SNP. Those oligos have specific sequences which are complementary to genomic regions and to universal primers, in addition, the LSO has a tagging sequence complementary to another oligo bound to a magnetic bead.

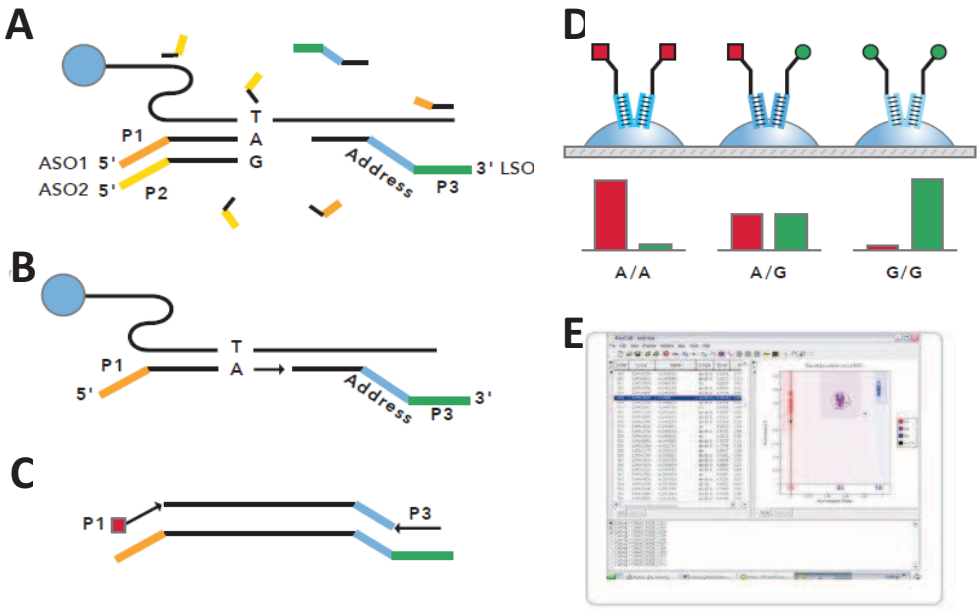

Figure 10. *Genotyping method. A: Hybridization; B: Extension and ligation; C: Amplification with universal primers P1/P2 y P3; D: Hybridization of the amplification products with the magnetic beads in the array; E: Read of the relative binding of the two SNP-specific primers. Address: Bead-tagging sequence; ASO: allele-specific oligo; LSO: locus-specific oligo; P1, P2, P3: primers and their complementary sequences.*

First, the hybridization between ASOs, LSO and genomic DNA must take place (Figure 10A). Then, extension and ligation (Figure 10B). Secondly, an amplification step takes place with three kings of primers (P1/P2 complementary to the ASOs; P3 complementary to the LSO) (Figure 10C). Then, the amplification products are hybridized in the array containing the SNP-specific magnetic beads. The relative binding of the SNP-specific primers is determined by a laser (Figure 10D), thus giving the genotype raw results (Figure 10E).

Genotyping was performed externally at the CIC Biogune Center of Bilbao, and the company Progenika provided the final raw results of the analysis.

Bioinformatic analysis

All the analysis from the raw genotyping results were performed with PLINK 1.07 software (Purcell et al. 2007)[4].

Quality control analysis

In every association study the first thing to do is to "clean" the data base removing low quality data. First, samples with low percentage of genotype results (lower than 90%) were removed. In this step, we removed 26 samples from the total 384 (6.8%). Second, the SNPs with a call rate (genotype results across the total samples) lower than 90% were removed, in our case this was 45 SNPs from the total of 492 (9.1%).

Hardy-Weinberg equilibrium

In population genetic studies, **Hardy-Weinberg (HW) equilibrium** establishes that the genetic composition of a population remains in equilibrium unless natural selection or other forces, such as mutations, take place (Mayo 2008). In our study, 5 SNPs showed deviation from equilibrium in the control group, and were removed. These SNPs can be found in the "table of genotyped SNPs" (Appendix).

Association study with obesity and phenotypes

For the association study, a logistic regression analysis was conducted with PLINK software, and OR was obtained with the 95% CI, for each SNP. The analysis was adjusted by sex and age. A total of 14 SNPs were found to be significantly

[4] http://pngu.mgh.harvard.edu/~purcell/plink/

Hardy-Weinberg equilibrium states that a population's genetic variation will remain constant between generations in the absence of disturbance factors (i.e. mutations).

associated with obesity. The Bonferroni correction method was used to counteract the problem of **multiple testing**. After the correction no significant results were found.

In the same way, a linear regression model adjusted by sex and age was applied to study the association between the SNPs and the studied phenotypes. In this case, we obtained the β coefficient, which indicates the number of units that the phenotype increases or decreases per risk allele. An additional adjustment by BMI was conducted in order to better characterize the observed associations. In every case, $p<0.05$ was considered to in order to consider statistical significance.

Statistical analysis

All continuous variables were expressed as mean ± standard error of the mean (SEM). Normal distribution of clinical parameters data was assessed by the Kolmogorov-Smirnov test. Insulin, HOMA-IR, total cholesterol, MMP-9 and tPAI-1 were logarithmically transformed to approximate normal distributions. Homogeneity of variances was estimated using the Levene test. Mean comparisons between obese and normal-weight children for continuous variables were compared by Student's t-test for unpaired samples. Correlations were estimated using Pearson's correlation coefficient for normally distributed variables and Spearman's for non-normally distributed variables. In every case statistical significance was considered when $p<0.05$. For these analysis, SPSS 15.00 (SPSS Inc.) was used.

The genotypic relative risk was assessed by comparing the obese group with the control group and calculating the OR and the 95% CI using a logistic regression analysis under the additive model implemented in PLINK after adjusting by age and sex. Linear regressions in the entire population, or separately per case and control group (data not shown in this thesis), were performed under the additive model to estimate the associations of each SNP with phenotypic parameters related with obesity and its complications.

Multiple testing can occur when one studies a set of statistical analysis simultaneously, in these occasions errors may happen due to false positives.

Chapter 1

LITERATURE REVIEW

Genetics of oxidative stress in obesity

Rupérez AI, Gil A & Aguilera CM

Submitted to *Clinical Science*

22nd October 2013

GENETICS OF OXIDATIVE STRESS IN OBESITY

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ABSTRACT

Obesity is a multifactorial disease characterised by the excessive accumulation of fat in adipose tissue and peripheral organs. Its derived metabolic complications are mediated by the associated inflammation, hypoxia and oxidative stress. Oxidative stress is due to the excessive production of free radicals or diminished antioxidant defences. Genetic variants such as single nucleotide polymorphisms in antioxidant defence system genes could alter the efficacy of these enzymes and ultimately the risk of obesity, thus, studies investigating the role of genetic variations in genes related to oxidative stress could be useful for better understanding the etiology of obesity and its metabolic complications. To our knowledge, this is the first review to revise the impact of genetic variations in oxidative stress genes including antioxidant enzymes, oxidative stress-producing systems and transcription factors and their relationship on obesity risk and its phenotypes.

INTRODUCTION

Obesity is increasing dramatically and has already become a major clinical challenge for health care systems worldwide (Shamseddeen *et al.* 2011). Obesity is a multifactorial disease, influenced by both genetic and environmental factors. The onset of obesity is due mainly to low energy expenditures (e.g., from exercise) combined with high caloric intake. This leads to an excessive accumulation of fat in the adipose tissue, accompanied by low-grade inflammation, hypoxia and oxidative stress.

Oxidative stress is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism. ROS include molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and the hydroxyl radical (OH^-). The controlled production of these molecules is known to help protect against microorganisms during infectious processes, as well as contribute to normal functions in the cell, including proliferation, differentiation and signalling (Gough *et al.* 2011). However, a non-physiological increase in ROS levels from excessive caloric intake, inflammation or hypoxia, or a decrease in the antioxidant capacity of the organism can lead to the aforementioned alterations.

The antioxidant defence system maintains ROS homeostasis in the cells. It comprises both endogenous and exogenous antioxidants. Endogenous antioxidants primarily include enzymes such as glutathione peroxidases (GPXs), catalase (CAT), paraoxonases (PONs), superoxide dismutases (SODs) and peroxiredoxins (PRDXs). These enzymes degrade ROS at different levels and in different compartments inside and outside of the cells. In addition, some of these enzymes require endogenous cofactors such as glutathione and lipoic acid in order to perform their ROS scavenging activities. Exogenous antioxidants include vitamins, carotenoids, polyphenols and trace elements such as selenium and zinc (reviewed by Da Costa *et al*. 2012).

It is known that genetic variations, such as single nucleotide polymorphisms (SNPs), can affect the functioning of antioxidant enzymes and increase the risk of certain diseases, such as cancer (Da Costa *et al.* 2012). However, to our knowledge, the impact of genetic variations in the genes associated with oxidative stress regulation has not been fully studied nor reviewed in the context of obesity. Detailed studies in this field could clarify the mechanisms involved in the development of the comorbidities of obesity, such as metabolic syndrome and insulin resistance. Thus, the aim of this review is to summarise the current knowledge about the association of genetic variations in antioxidant defence

system genes, oxidative stress producing systems and related transcription factors with obesity risk and phenotypes.

In the future, the characterisation of these SNPs in obese patients could contribute to the development of controlled antioxidant therapies that would be potentially beneficial for the treatment of obesity-derived metabolic complications.

METHODOLOGY

We conducted a systematic review of the literature by using PubMed database. The following phrases were included in the process: 1) "obesity" AND "polymorphism" AND "oxidative stress" limited to human studies gave 47 results; 2) "obesity" AND "polymorphism" AND "oxidative stress" limited to animal studies gave 8 results; 3) "obesity" AND "gene expression" AND "adipose tissue" AND "oxidative stress" limited to human studies gave 28 results; 4) "obesity" AND "gene expression" AND "adipose tissue" AND "oxidative stress" limited to animal studies gave 71 results; 5) "obesity" AND "mechanisms" AND "gene" AND "oxidative stress" limited to human studies gave 31 results; and 6) "obesity" AND "mechanisms" AND "gene" AND "oxidative stress" limited to animal studies gave 30 results. A total of 215 results in English were obtained and titles and abstracts were revised to select a total of 73 articles that were read in full. Additional articles not found in this search were identified by exploring references in key articles.

OXIDATIVE STRESS IN OBESITY

The adipose tissue is an endocrine organ that produces a variety of molecules, including adipokines, such as adiponectin and leptin, and cytokines, such as tumour necrosis factor alpha (TNFα) and interleukins 1β (IL-1β) and 6 (IL-6) (Balistreri *et al.* 2010; Trayhurn *et al.* 2004). It is well known that adipose tissue in obese individuals undergoes many pathological changes due to the accumulation of fat, such as inflammation, hypoxia and increased oxidative stress (Bondia-Pons *et al.* 2012; Trayhurn 2013). Upon the accumulation of excessive fat, the adipokine secretion profile becomes altered, and peripheral tissues are affected, contributing to the appearance of health problems such as dyslipidemia, hypertension, insulin resistance, diabetes and atherosclerosis (Figure 1.1).

The higher fat and carbohydrate intakes associated with obesity may be responsible in part for the enhanced ROS production, due to the saturation of the

electron transport chain. Free fatty acids (FFAs) have had this effect in mouse models (Furukawa *et al.* 2004), and in humans, FFAs generate high H_2O_2 levels in the mitochondria (Anderson *et al.* 2009). Thus, the link between obesity and enhanced oxidative stress might be due to the hyperglycaemia, high circulating FFA, decreased antioxidant defences and chronic inflammation associated with obesity. Indeed, in obese humans, indicators of cellular and systemic oxidative stress have been found in many studies. Levels of plasma thiobarbituric acid reactive substances (TBARS) and urinary 8-epi-prostaglandin F2α (8-epi-PGF2α) were augmented in obese individuals (Furukawa *et al.* 2004; Keaney *et al.* 2003). A study conducted in severely obese children found similar results, with higher 8-isoprostane F2α and malondialdehyde (MDA) plasma concentrations, as well as increased nitric oxide production, as reflected by higher nitrite, nitrate and nitrotyrosine values, in obese children (Codoñer-Franch *et al.* 2011). The activities of antioxidant enzymes such as glutathione peroxidase and catalase have also been observed to be lowered in obesity, as will be described below.

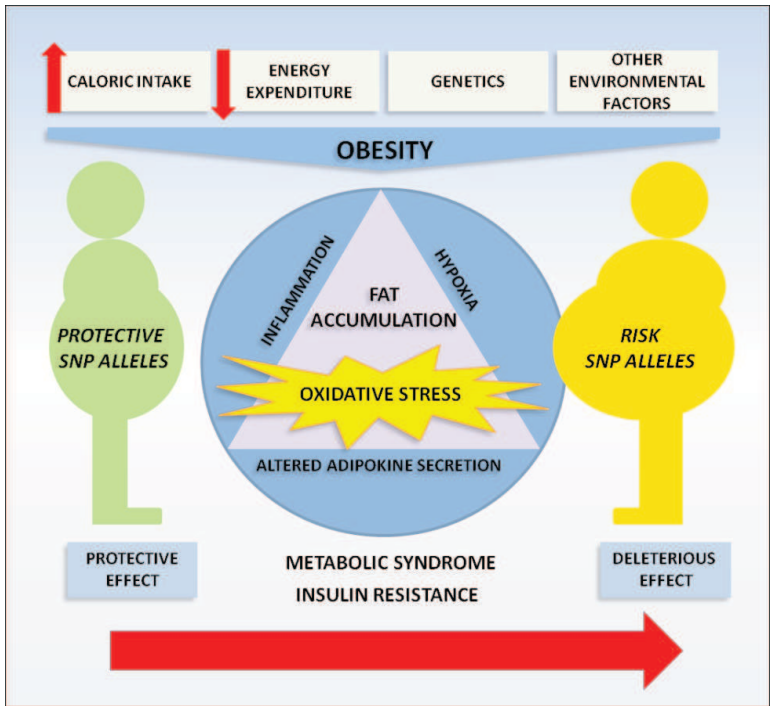

Figure 1.1. *Schematic diagram of the multifactorial character of obesity. These factors influence the development of obesity and its associated comorbidities by altering adipokine secretion, hypoxia and inflammation with associated oxidative stress. The presence of certain SNPs reviewed here can increase the risk of obesity and its comorbidities, further worsening the metabolic profile. SNP: single nucleotide polymorphism.*

In addition to ROS produced by caloric intake, cells also have ROS-producing systems for physiological processes, such as the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) complex that produces ROS in response to insulin and cytokines (like TNFα) as part of a signal-transducing system (Krieger-Brauer *et al.* 1995). The alteration of these systems can generate additional quantities of ROS and contribute to higher oxidative stress levels.

The enhanced oxidative stress associated with obesity leads to the oxidation of proteins, lipids and DNA, and eventually, to alterations in the modulation of gene expression and signalling pathways (Finkel 2011). Indeed, these alterations in cellular and tissue components contribute to chronic inflammation, and thus to the development of diseases such as obesity and insulin resistance (Houstis *et al.* 2006). In fact, chronic oxidative stress is known to induce inflammation, and obesity is considered a disease of chronic low-grade inflammation (Gregor *et al.* 2011).

The role of transcription factors in the development of obesity comorbidities is gaining attention. Peroxisome proliferator activated receptor gamma (PPARγ) and PPARγ coactivator-1α (PGC1α) are very well-known transcription factors in the adipose tissue, and they are also implicated in cellular responses against oxidative stress. In the present review, we will focus on the known variations in these genes and their impact on the risk of obesity insulin resistance. Nuclear erythroid factor 2-like 2 (NRF2), one of the most important transcription factors in the oxidative stress response, will also be considered with respect to its role in ROS responses and obesity.

ENZYMATIC ANTIOXIDANT DEFENCE GENES

Glutathione peroxidases

The GPX family is composed of at least six isoenzymes and constitutes one of the main antioxidant defence systems, using glutathione to degrade hydrogen peroxide (Margis *et al.* 2008). GPX1 is the most abundant isoenzyme, ubiquitous in the intracellular fraction and formed by four 22-kDa subunits, each carrying one selenocysteine.

Cellular and extracellular GPX activity was shown to be lower in the adipose tissue of obese rats (Asayama *et al.* 2001). In a study carried out with obese mice, the mRNA expression of *GPX1* was increased after caloric restriction (Lijnen *et al.* 2012). Moreover, in another study involving obese mice, the expression of *GPX3*

was found to be selectively decreased in adipose tissue and plasma; the same effect was achieved by treating mice with either both TNFα or hypoxia. The use of antioxidants and the anti-diabetic drug rosiglitazone both succeeded in restoring *GPX3* expression in adipose tissue, improving the insulin resistance phenotype and attenuating the inflammatory gene expression pattern (Lee *et al.* 2008). Another study that supports GPX3 activity against obesity showed that oestrogen receptor α activates *GPX3* transcription and that this mediates its fat mass reducing effects (Lundholm *et al.* 2008).

Regarding genetic variations, SNPs associated with obesity and insulin resistance have only been described for the *GPX1* gene (Figure 1.2, Table 1.1). The *GPX1* gene harbours a well-known missense polymorphism (C to T substitution) at nucleotide 594 that results in the substitution of leucine for proline at codon 198 of the protein (Pro198Leu; rs1050450). Many studies have shown the Leu allele to be associated with worse outcomes for oxidative stress, central obesity and insulin resistance, with some sex-related differences. Male Leu allele (T) carriers had higher metabolic syndrome prevalence, demonstrating higher waist-hip ratios, triglycerides (TAG), insulin, homeostasis model assessment of β-cell function (HOMA-β) and systolic and diastolic blood pressures (Kuzuya *et al.* 2008). Women carrying the same allele showed higher body fat mass, insulin and homeostasis model assessment of insulin resistance (HOMA-IR). In an intervention study, the authors showed that nutritional supplementation with selenium from Brazil nuts was associated with higher DNA damage in Leu carriers (Cominetti *et al.* 2011). Carriers of the Leu allele have also been shown to have significantly higher levels of lipoperoxides and MDA in low-density lipoproteins (LDL) (Shuvalova *et al.* 2010).

It was shown that the combination of Pro198Leu SNP with the copy number variant (CNV) Ala^5/Ala^6 at codon7-11 decreases the activity of the enzyme by 40% *in vitro* (Hamanishi *et al.* 2004). In the same study, it was demonstrated that the combination of two other SNPs (-602A/G, 2C/T) decreased the transcriptional activity of *GPX1* by 25%.

These data suggest that the Leu allele is associated with lower GPX activity and a subsequent higher oxidative stress from ROS, thus generating worse outcomes in obesity-associated phenotypes.

Catalase

CAT is one of the most important antioxidant enzymes in the cell, located in the peroxisomes. It degrades any hydrogen peroxide that exceeds the physiological levels. *CAT* expression was increased after caloric restriction in obese mice (Lijnen *et al.* 2012), and its erythrocyte activity was lower in children with insulin resistance and obesity (Shin *et al.* 2006; Ruperez *et al.* 2013).

Its genetic variation has traditionally been studied for diseases other than obesity. Due to this fact, our group recently published a study conducted on obese children that showed the association of some SNPs located in the *CAT* promoter with obesity (Ruperez *et al.* 2013) (Figure 1.2, Table 1.1). We found that the presence of the rare SNP variants rs769214 (-844A/G), rs7943316 (-89T/A) and rs1049982 (-20C/T) was significantly and positively associated with prepubertal obesity. All of these SNPs were in linkage disequilibrium (LD) and formed a haplotype that had been previously described as associated with lower *CAT* expression in human cell lines under high oxidative stress (Wang *et al.* 2010). The association between these SNPs and the principal insulin resistance and obesity markers was also studied, and we found that SNP rs769214 is associated with significantly higher weight, body mass index (BMI) *z*-score and adipocyte fatty acid-binding protein (A-FABP), as well as with a higher plasma insulin concentration (not significant), without any observed effect on erythrocyte catalase activity. Another variant investigated was the SNP –262C/T in the 5' UTR region of the *CAT* gene. Several studies demonstrated that the T allele was associated with lower CAT enzyme activity (Ahn *et al.* 2005; Nadif *et al.* 2005; Bastaki *et al.* 2006), whereas those results were rejected by others (Forsberg *et al.* L).

These results suggest that CAT activity and expression are involved in the defence mechanisms against obesity-derived metabolic complications. The presence of the described SNPs could lead to lower *CAT* transcriptional activity and thus to lower CAT expression levels and activity in the cell. This would further contribute to cellular oxidative stress and its effects on cell dysfunction by altering signalling cascades or increasing the damage to macromolecules by oxidation.

Paraoxonases

The PON family consists of three antioxidant isoenzymes. PON1 and PON3 are expressed mainly in the liver and kidneys and are found bound to high-density lipoproteins (HDL) in the circulation. They inhibit the lipid peroxidation of the LDL

and HDL particles in plasma. PON2 is a more ubiquitous membrane-bound form that is found in a variety of tissues. Regarding alterations in PON expression in obesity, only one study has been conducted, in pigs, where *PON3* mRNA expression in fat tissue was positively correlated with subcutaneous, visceral and total body fat weight, indicating a role for PON3 in obesity (Labrecque *et al.* 2009).

PON1 activity is known to be influenced by environmental factors such as age, diet or medications, but the main cause of variation is genetics (Costa 2011). Two missense SNPs in the *PON1* gene, Q192R (rs662) and L55M (rs854560), have been traditionally studied (Figure 1.2, Table 1.1). Individuals with the 192RR genotype have higher paraoxon-degrading activity (Adkins *et al.* 1993), while 55LL individuals exhibit increased serum PON1 concentrations (Garin *et al.* 1997). A study conducted in Portuguese women showed an association of the R allele with a higher risk of obesity (Veiga *et al.* 2011), whereas a study in Mexican adults revealed no association between the variant and risk of obesity (Martinez-Salazar *et al.* 2011). Another study failed to find any association of the SNP with obesity in adolescents (Baráth *et al.* 2006). Our group recently published a study carried out in prepubertal children that also confirmed the lack of an effect of Q192R on childhood obesity risk (Rupérez *et al.* 2013). In this study, we described a novel *PON1* SNP, rs854566, that was found to be associated with protection from obesity in children, perhaps due to an increase in PON1 lactonase activity, although PON1 activities did not show any differences between obese and normal-weight subjects (Figure 1.2).

Peroxiredoxins

PRDXs are a family of six thioredoxin-dependent peroxidases that degrade H_2O_2 in the cell. Recent studies have clearly shown that PRDXs contribute to ROS signalling, regulating cell proliferation, differentiation and apoptosis. PRDX3 is located exclusively in the mitochondria, where it scavenges up to 90% of the H_2O_2 produced in this organelle, followed by scavenging by GPX1 and GPX4 (Cox *et al.* 2010). Taking into account that mitochondrial respiration is the principal ROS producer, PRDX3 is considered highly important in terms of antioxidant defences and redox status regulation.

PRDX3 levels have been observed to be decreased in the adipose tissue of obese mice and humans (Huh *et al.* 2012). In the same study, *PRDX3* knock-out mice exhibited increased fat mass as well as increased adipogenic and lipogenic gene expression in adipose tissue. Additionally, increased superoxide levels and

protein carbonylation were observed in mitochondria, together with defects in mitochondrial biogenesis. In addition, adiponectin was downregulated, and plasminogen activator inhibitor (PAI) was upregulated, in accordance with the presence of impaired glucose tolerance and insulin resistance observed in *PRDX3* knock-down adipocytes.

The impact of *PRDX3* genetic variations on obesity has been investigated in only one nutrigenomic study, in which it was found that four SNPs in the *PRDX3* gene and the haplotype they formed were associated with higher BMI and obesity in Japanese people, when combined with a high-fat diet (HFD) (Hiroi *et al.* 2011) (Figure 1.2, Table 1.1). The SNPs rs3740562 (A/G), rs2271362 (C/T), rs7768 (G/C) and rs3377 (A/C) were significantly associated with BMI after a multiple testing Bonferroni correction, whereas rs1553850 (A/T) was not. The haplotypes A-A-T-G-A and T-G-C-C-C also showed a significant association with decreased and increased BMI, respectively. As a HFD induces ROS production, Hiroi *et al.* investigated its possible role in these associations. The study of the interactions between the genotypes and haplotypes and dietary fat intake revealed that these genetic associations could only be observed in the group with the high-fat intake. Moreover, the association of the genotypes with higher BMI was observed only in the high-fat intake group. Altogether, these findings indicated a role for *PRDX3* genetic variations and fat intake in the modulation of BMI and obesity risk.

More studies are needed to investigate the effects of these and other SNPs on enzyme levels and activities to elucidate the link between their presence and a higher risk of obesity.

Superoxide dismutases

The three members of the SOD family are the first line of defence against ROS, eliminating the strong superoxide radical and producing H_2O_2 that can then be degraded by CAT, GPXs and PRDXs (Zelko *et al.* 2002). CuZn-SOD (SOD1) is a homodimer localised in the cytosol, Mn-SOD (SOD2) is a tetramer localised in the mitochondria, and the extracellular tetramer CuZn-SOD (SOD3 or EC-SOD) is localised exclusively in extracellular spaces. MnSOD is one of the most important antioxidant enzymes because most superoxide is produced in the mitochondria.

EC-SOD levels have been observed to increase in the white and brown adipose tissue and in the plasma of obese mice. In the same study, TNFα and IL-1β levels were also observed to be higher in white adipose tissue, which could be interpreted as an adaptation by the adipose tissue to the enhanced oxidative stress

associated with obesity (Nakao *et al.* 2000). However, in a study of type 2 diabetic patients, EC-SOD levels were shown to be reduced and inversely related to BMI and HOMA-IR (Adachi *et al.* 2004).

The best-known *SOD2* SNP is rs4880 (C/T). It is located in the second exon, and its presence generates a change in the 16th amino acid in the mitochondrial targeting sequence of the protein from alanine to valine (Shimoda-Matsubayashi *et al*. 1996) (Figure 1.2, Table 1.1). The Val-MnSOD variant has been associated with the arrest of MnSOD in the inner membrane and lower MnSOD homotetramer formation in the mitochondrial matrix, together with a lower efficiency of the enzyme in dismutating superoxide into H_2O_2 (Sutton *et al.* 2003). Strikingly, both alleles in their homozygous form have been found to be associated with an increased risk for a variety of diseases, perhaps due to the increased superoxide or hydrogen peroxide levels in ValVal and AlaAla subjects, respectively. However, only the Val allele was found to be associated with a higher risk of obesity in the elderly (Montano *et al.* 2009). In addition, the presence of the ValVal genotype was related to higher levels of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α and interferon gamma (IFN-γ), and lower levels of IL-10 (Montano *et al.* 2012). In contrast, Val allele carriers from a healthy cohort showed lower baseline levels of DNA damage (Caple *et al.* 2010). Another study found that the ValVal genotype was more frequent among obese children with non-alcoholic steatohepatitis than those without the disease, although this difference was not significant (El-Koofy *et al.* 2011).

These findings indicate that the role of superoxide in the origin of the metabolic complications of obesity should be investigated.

THE ROS PRODUCER: NADPH oxidase

One of the most important ROS producers in cells is the NADPH oxidase complex, which generates O_2^-, and subsequently other ROS such as H_2O_2, during the phagocyte respiratory burst (DeCoursey *et al.* 2005). However, its activity is not limited to phagocytes, as other cells use NADPH oxidase-generated ROS as signalling mechanisms. It is known that insulin and cytokines act on this enzymatic complex, stimulating H_2O_2 production and providing a link between ligand binding and the intracellular redox state contributing to intracellular signalling cascades (Krieger-Brauer *et al.* 1995). This complex is formed from 6 subunits: p22phox and gp91phox, which form cytochrome b_{558}; p47phox; p67phox; p40phox; and rac. This enzymatic complex generates free radicals from oxygen and NADPH.

The SNP -930A/G in the promoter of the p22phox subunit gene was found to be associated with higher p22phox expression and NADPH oxidase activity in phagocytic cells from hypertensive patients carrying the GG genotype (San José *et al.* 2004) (Figure 1.2, Table 1.1). The higher NADPH oxidase activity resulted in higher ROS production, which in turn increased the risk of insulin resistance (Eriksson 2007). Along these lines, the GG genotype was associated with higher HOMA-IR and insulin but not with obesity in a cohort of obese and normal weight Spanish subjects (Ochoa *et al.* 2008). Another SNP in the p22phox subunit is 242C/T (Figure 1.2). Japanese type 2 diabetic patients carrying the T allele of this SNP showed a significantly lower intima media thickness (IMT) and lower 8-hydroxy-2'-deoxyguanosine (8-OHdG) values (not significant), whereas the non-diabetic T allele carriers were protected against insulin resistance, exhibiting lower HOMA-IR and fasting plasma insulin values (Hayaishi-Okano *et al.* 2003). However, another study reported that the CC genotype conferred protection against diabetes mellitus and obesity and was associated with lower fasting plasma glucose levels and waist circumference in hypertensive patients (Schreiber *et al.* 2012). Other studied SNPs include rs7195830 (C allele) and rs12709102 (T allele), which were associated with a higher risk of obesity in women (Kim *et al.* 2012).

All of these findings taken together indicate that higher NADPH oxidase activity and the concomitant ROS production could act in modulating the insulin signalling pathway. The genetic variations in the genes of NADPH oxidase subunits should be further investigated to better understand their impact on enzymatic activity and the consequences on insulin signalling. This phenomenon could be a link between obesity and insulin resistance. Knowing the genotype of obese patients could help in treating them against further damaging metabolic complications.

ROS RESPONSE MECHANISMS: TRANSCRIPTION FACTORS

PPARγ

The nuclear hormone transcription factor PPARγ regulates adipogenic differentiation and lipid metabolism. Its expression is increased in the adipose tissue of obese individuals (Vidal-Puig *et al.* 1997; Berhouma *et al.* 2013). It binds to lipophilic ligands such as poly-unsaturated fatty acids (PUFAs), prostaglandin derivatives and oxidised fatty acids (Zielekiak *et al.* 2008). Some studies have shown that PPARγ plays a role in the regulation of the antioxidant response to

ROS, although the results are inconsistent. In a study in mice, it was shown that the adipose tissue-specific loss of an allele of PPARγ, with the subsequent loss of activity, was associated with more resistance to paraquat-induced oxidative stress. This was at least partially mediated through the upregulation of ROS scavenging genes, including *GPX1*, glutathione reductase, *PRDX3*, *SOD2* and *CAT*, and the upregulation of the ROS responding transcription factor *FOXO3A* in adipose tissue (Luo *et al.* 2008). This study concluded that reduced PPARγ activity in adipose tissue has beneficial effects. However, in another study, the authors showed that the activation of PPARγ by its ligands decreased TNFα or glucocorticoid-induced ROS production in human adipocytes (Houstis *et al.* 2006). In a previous study, Itoh *et al*. hypothesised that oxidative stress could exert some of its effects on intracellular signalling through PPARγ, and indeed, they showed that *PPARγ* expression was downregulated by H_2O_2 and lysophosphatidyl choline, which is the major constituent of oxidised LDL (ox-LDL), and TNFa (Itoh *et al.* 1999).

The best-known SNP in the *PPARγ* gene is rs1801282, which generates an amino acid change in the protein at codon 12 from proline to alanine (Pro12Ala) (Figure 1.2, Table 1.1). The effect of this SNP has been investigated in many previous studies, with inconsistent results. The presence of the Ala allele was shown to decrease receptor-mediated transcription activity and to be associated with a lower BMI and increased insulin sensitivity (Deeb *et al.* 1998). However, in a recent meta-analysis that included almost 50,000 subjects, the authors showed that individuals carrying the Ala allele have an increased BMI (+0.065 kg/m^2), with a stronger effect in Caucasians (Galbete *et al.* 2012).

Although it is becoming clear that PPARγ has a role in ROS clearance from adipose tissue, this paradox has yet to be investigated, to clarify whether the activation of PPARγ decreases ROS production or increases ROS scavenging. Moreover, the association of the Ala allele with obesity needs to be clarified in a controlled experimental setting while carefully studying ROS production.

PGC1α

PGC1α is a transcriptional co-activator of PPAR α and γ and controls mitochondrial biogenesis, adaptive thermogenesis, oxidative metabolism and glucose homeostasis. In these ways, it increases the oxidative metabolism that will lead to oxidative stress. However, PGC1α also induces the expression of ROS detoxifying enzymes, thus allowing for enhanced oxidative metabolism while controlling the associated ROS production (Austin *et al.* 2012; Valle *et al.* 2005).

The PGC1α locus harbours the SNP rs8192678, which results in an amino acid substitution of glycine to serine at position 482 (Gly482Ser) (Figure 1.2, Table 1.1). Fanelli *et al*. found that the Gly482Ser variant was associated with HOMA-IR in obese non-diabetic subjects (Fanelli *et al.* 2005). In another study, the Gly482Ser variant was significantly associated with a lower BMI, waist and hip circumference and total body fat, but only in women (Esterbauer *et al.* 2002). This SNP was not found to be associated with obesity or type 2 diabetes in overweight non-diabetic Chinese individuals, but it was associated with high insulin, HOMA-IR and waist-hip ratios, as well as with TBARS in hyperglycaemia (Weng *et al.* 2010). Because adiponectin is under the transcriptional control of PPARγ, a target of PGC1α, Okauchi *et al.* studied the effects of the Gly482Ser variant on adiponectin plasma levels and found lower adiponectin concentrations in type 2 diabetic men, but not in women, carrying the polymorphism (Okauchi *et al.* 2008). In this study, they ruled out the possibility that this variant is a functional polymorphism, and they suggested that the causative SNP could be in LD with the common Gly482Ser variant. Along these lines, a previous functional study had already determined that neither the Gly482Ser nor Trp612Met variants of *PGC1α* affected the functionality of the protein regarding its co-activator activity on PPARγ2 (Nitz *et al.* 2007). A study conducted on the Gly482Ser polymorphism showed a basal association for the SerSer genotype with higher HOMA-IR and insulin concentrations, but an intervention with an 8-week low-calorie diet reduced the risk level to that of non-carriers (Goyenechea *et al.* 2008).

These results indicate that the Gly482Ser variant has an effect on obesity-associated comorbidities such as insulin resistance, although it is most likely another SNP that is responsible for these effects.

Nrf2

The transcription factor NRF2 regulates cellular responses to oxidative stress and other endogenous and exogenous stresses. Its role in obesity, type 2 diabetes and metabolic syndrome has already been investigated in many studies using animal models (reviewed by Chartoumpekis *et al*. 2013). NRF2 is regulated mainly through its binding to Kelch-like ECH-associated protein 1 (KEAP1) in the cytoplasm, which leads to its proteasomal degradation. The NRF2/KEAP1 pathway responds to oxidative stress via the control of several antioxidant defence gene expressions harbouring the Antioxidant Response Element (ARE) sequence in their promoter (Figure 1.2).

Along with its role in the response against oxidative stress, NRF2 also modulates adiposity and adipogenesis. In fact, protein levels of NADPH quinone oxidoreductase (NQO1), which is under the transcriptional control of NRF2, increase during the initial stages of the adipogenic differentiation process (days 1-3). Moreover, in addition to NQO1, NRF2 and KEAP1, mRNA levels are also increased in differentiated adipocytes (days 11-14) (Vomhof-DeKrey *et al.* 2012). This finding has been further confirmed by Hou *et al.*, who found that the lack of NRF2 in 3T3-L1 cells blocked adipogenic differentiation by suppressing CCAAT/enhancer-binding protein beta (CEBPβ) expression, which is needed to trigger the differentiation process (Hou *et al.* 2012). In contrast, Chartoumpekis *et al.* observed lower NRF2 abundance in the nucleus during adipogenesis, which they hypothesised could lead to the higher ROS levels needed for the differentiation process (Chartoumpekis *et al.* 2011-a).

Among the *in vivo* studies on the role of NRF2 in obesity, the main conclusion from knock-out studies in mice is that the targeted disruption of *NRF2* decreases adipose tissue mass and protects mice from long-term HFD-induced obesity (Chartoumpekis *et al.* 2011-b; Pi *et al.* 2010). *NRF2* knock-out mice are partially protected from HFD-induced obesity and insulin resistance. This effect may be due to the effect of fibroblast growth factor 21 (FGF21); the mRNA levels of FGF21 in the liver and white adipose tissue were elevated in *NRF2* knock-out mice. The opposite effect, i.e., lower FGF21 mRNA levels, was observed when NRF2 was over-expressed (Chartoumpekis *et al.* 2011-b). In another study, Shin *et al*. observed the same effects of the *NRF2* knock-out. However, they also tested the pharmacological activation of NRF2 by 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im) and observed that it also protected from obesity by facilitating higher energy expenditure (Shin *et al.* 2009). One of the main questions arising from these findings concerns which tissue is responsible for the NRF2 effect. One experiment with myeloid cells showed that deficiency in this tissue did not protect mice from HFD-induced adipose tissue inflammation and insulin resistance (Meher *et al.* 2012). As Chartoumpekis *et al.* state in their review, Cre-loxP system experiments with tissue-specific knock-out models would help further clarify this issue. To our knowledge, only one study of NRF2 in humans has been published, demonstrating how the NRF2 pathway was enriched in individuals with high-fat percentages (Das *et al.* 2011).

Many SNPs have been described for the *NRF2* gene in both mice and humans (Cho *et al.* 2013), although the association of *NRF2* SNPs and obesity has not been studied. However, other diseases, such as pulmonary disease, breast cancer, and

gastrointestinal and autoimmune disorders, have shown different associations with *NRF2* SNPs (reviewed by Cho *et al.* 2013). The SNP at position −178C/A conferred lower promoter activity in carriers of the A allele (Marzec *et al.* 2007). Interestingly, all evidence indicates that promoter and intronic SNPs are the ones that most show associations with the conditions studied, with no exonic SNP described so far.

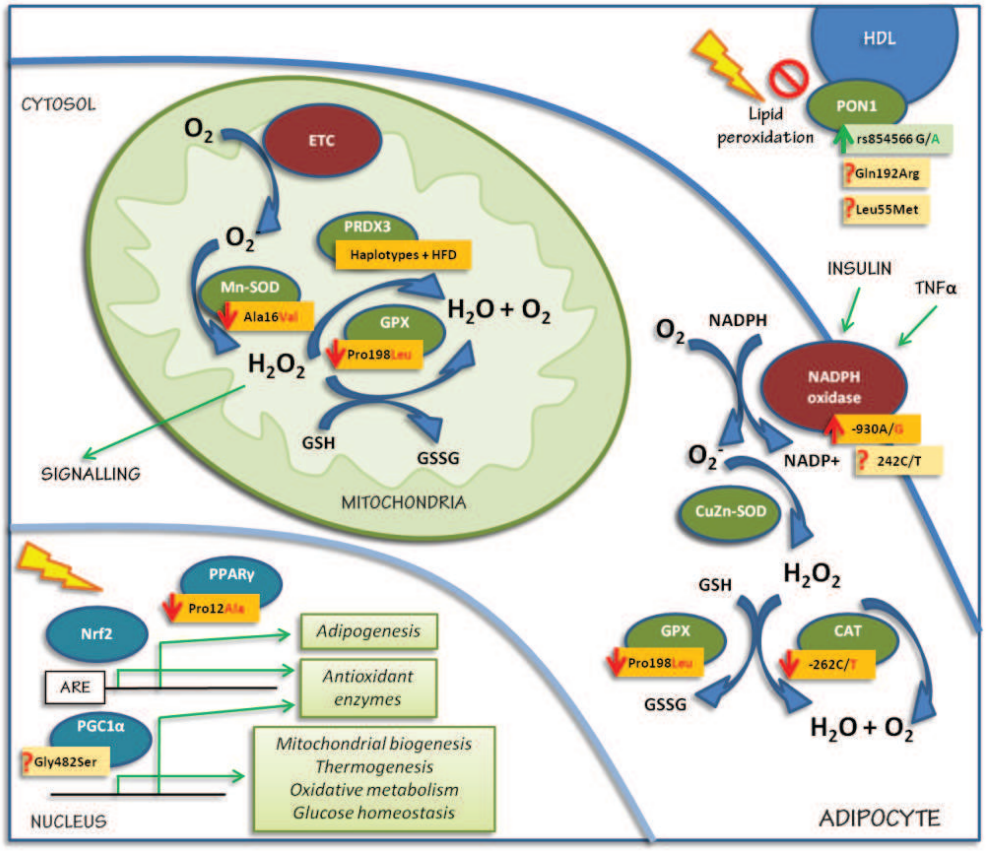

***Figure 1.2**. The effects of the main known SNPs of the antioxidant defence system genes in the cell on obesity. Orange and green boxes indicate higher or lower obesity risk associated with the SNP, respectively. Red and green arrows matching the colour of an allele of the SNP indicate higher or lower enzyme activity associated with that allele. Question marks indicate conflicting results. Long thin green arrows indicate activation. CAT: catalase; CuZn-SOD: copper-zinc superoxide dismutase; ETC: electron transport chain; GPX: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; HDL: high-density lipoprotein; HFD: high-fat diet; Mn-SOD: manganese superoxide dismutase; NADPH: nicotinamide adenine dinucleotide phosphate; NRF2: nuclear erythroid factor 2 like 2; $O_2^{\bullet-}$: superoxide; PGC1α: peroxisome proliferator-activated receptor γ coactivator-1α; PON1: paraoxonase 1; PPARγ: peroxisome proliferator activated receptor gamma; PRDX3: peroxiredoxin 3; TNFα: tumour necrosis factor alpha.*

CONCLUSIONS

The study of genetic variants in antioxidant defence genes, as well as in the genes of enzymes involved in the generation of ROS, could assist in better understanding the role of antioxidant defences in protecting against obesity and its derived metabolic complications. There are data supporting the fact that obesity occurs along with enhanced ROS production, either due to misbalanced ROS scavenging systems or to enhanced oxidative stress production in cells. These effects can be due to excessive caloric intake and saturation of the electron transport chain, as well as to free radical generation from cellular systems such as the NADPH oxidase complex, in response to the altered insulin or cytokine production that is characteristic of obesity.

This review summarises the studies that have been carried out to unravel the role of genetic variants in antioxidant defence enzymes and other important oxidative metabolism mediators in increasing the risk of obesity or its close comorbidities, such as insulin resistance.

More studies of the genetic variation in the antioxidant defence system are needed to clarify the associations reviewed here, as well as more functional studies concerning these SNPs and their possible impact on enzyme expression levels and activities.

LIMITATIONS

The present review has included only antioxidant enzymes and transcription factors of high importance with known genetic variations that affect their function and oxidative stress status. NRF2 was an exception, lacking any SNP associated with obesity, but it was included due to its importance in the regulation of ROS response mechanisms.

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DISCLOSURE STATEMENT

The authors have nothing to disclose.

Table 1.1. SNPs from oxidative stress-related genes and their impact on obesity outcomes.

Gene	Analysed variant (dbSNP)	Other designations	Alleles 1/2	Outcome	Reference
GPX1	rs1050450	594C/T Pro198Leu	C/T	Leu male carriers have higher waist-hip ratio, TAG, insulin, HOMA-β, SPB, DPB Leu female carriers have higher body fat mass, insulin and HOMA-IR	Kuzuya 2008
				Leu carriers show higher DNA damage after Se supplementation	Cominetti 2011
				Leu carriers have higher lipoperoxides and MDA in LDL	Shuvalova 2010
GPX1		Ala^5/Ala^6 + Pro198Leu		40% decrease in GPX1 activity	Hamanishi 2004
GPX1		-602A/G + 2C/T		25% decrease in transcriptional activity	Hamanishi 2004
CAT	rs769214	-844A/G	A/G	Associated with prepubertal obesity, higher weight, BMI *z*-score, A-FABP	Ruperez 2013
CAT	rs7943316	-89T/A	A/T	Associated with prepubertal obesity	Ruperez 2013
CAT	rs1049982	-20C/T	C/T	Associated with prepubertal obesity	Ruperez 2013
CAT		-844G/-89A/-20T		Lower CAT expression under high oxidative stress conditions	Wang 2010
CAT	rs1001179	-262C/T	C/T	Lower CAT activity	Ahn 2005, Nadif 2005, Bastaki 2006
				No effect on CAT activity	Forsberg L
PON1	rs662	Q192R	G/A	192RR individuals show higher activity degrading paraoxon	Adkins 1993
				R allele associated with higher obesity risk	Veiga 2011
				No association with obesity in Mexican adults	Martinez-Salazar 2011

Gene	Analysed variant (dbSNP)	Other designations	Alleles 1/2	Outcome	Reference
PON1	rs662	Q192R	G/A	No association with obesity in adolescents	Baráth 2006
				No association with obesity in prepubertal children	Rupérez 2013
PON1	rs854560	L55M	A/T	55LL have increased serum PON1 concentrations	Garin 1997
PON1	rs854566		G/A	Protection against prepubertal childhood obesity and lactonase activity	Rupérez 2013
PRDX3	rs3740562*		A/G	Associated with higher BMI in Japanese adults when combined with high-fat diet	Hiroi 2011
PRDX3	rs2271362*		C/T	Associated with higher BMI in Japanese adults when combined with high-fat diet	Hiroi 2011
PRDX3	rs7768*		G/C	Associated with higher BMI in Japanese adults when combined with high-fat diet	Hiroi 2011
PRDX3	rs3377*		A/C	Associated with higher BMI in Japanese adults when combined with high-fat diet	Hiroi 2011
SOD2	rs4880	Ala16Val	C/T	Val variant associated with MnSOD arrest in the inner mitochondrial membrane and lower dismutase efficiency	Sutton 2003
				Val allele associated with higher obesity risk in elderly	Montano 2009
				Val allele show higher levels of IL-1, IL-6, TNFα, IFNγ and lower levels of IL-10	Montano 2012
				Val allele carriers show lower DNA damage levels	Caple 2010
p22phox	rs9932581	-930A/G	A/G	GG carriers have higher p22phox expression and NADPH oxidase activity	San Jose 2004
				GG carriers have higher HOMA-IR and insulin but not higher obesity risk	Ochoa 2008

Gene	Analysed variant (dbSNP)	Other designations	Alleles 1/2	Outcome	Reference
p22phox	rs4673	242C/T	C/T	T allele type 2 diabetic carriers have lower IMT and 8-OHdG values, non-diabetic carriers have lower HOMA-IR and insulin	Hayaishi-Okano 2003
				CC carriers protected against diabetes and obesity, with lower plasma glucose levels and WC in hypertensive patients	Schreiber 2012
p22phox	rs7195830		C/T	C allele associated with higher obesity risk in women	Kim 2012
p22phox	rs12709102		T/C	T allele associated with higher obesity risk in women	Kim 2012
PPARγ	rs1801282	Pro12Ala	C/G	Ala allele decreases receptor mediated transcriptional activity and is associated with lower BMI and increased insulin sensitivity	Deeb 1998
				Ala allele associated with increased BMI (+0.065 kg/m^2 per allele)	Galbete 2012
PGC1α	rs8192678	Gly482Ser	G/A	Ser allele associated with HOMA-IR in obese subjects Ser variant associated with lower BMI, waist and hip circumference and total body fat in women	Fanelli 2005 Esterbauer 2002
				Not associated with obesity or T2D in overweight Chinese subjects but associated with high insulin, HOMA-IR and waist-hip ratio, as well as TBARS, in patients with hyperglycaemia	Weng 2010
				Ser male diabetic carriers have lower adiponectin plasma levels	Okauchi 2008
				Ser carriers have higher HOMA-IR and insulin concentrations, but 8-week low calorie diet reduces the risk	Goyenechea 2008

* And their haplotype; Alleles; 1: major, 2: minor. CAT: catalase; GPX: glutathione peroxidase 1; p22phox: cytochrome b-245 alpha polypeptide; PON1: paraoxonase 1; PRDX3: peroxiredoxin 3; PGC1α: peroxisome proliferator-activated receptor γ coactivator-1α; PPARγ: peroxisome proliferator activated receptor gamma; SOD2: manganese superoxide dismutase.

REFERENCES

Adachi, T., Inoue, M., Hara, H., Maehata, E. and Suzuki, S. (2004) Relationship of plasma extracellular-superoxide dismutase level with insulin resistance in type 2 diabetic patients. J. Endocrinol. **181**, 413-417

Adkins, S., Gan, K.N., Mody, M. and La Du, B.N. (1993) Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. Am. J. Hum. Genet. **52**, 598-608

Ahn, J., Gammon, M.D., Santella, R.M., Gaudet, M.M., Britton, J.A., Teitelbaum, S.L., Terry, M.B., Nowell, S., Davis, W., Garza, C., Neugut, A.I. and Ambrosone, C.B. (2005) Associations between breast cancer risk and the catalase genotype, fruit and vegetable consumption, and supplement use. Am. J. Epidemiol. **162**, 943-952

Anderson, E.J., Lustig, M.E., Boyle, K.E., Woodlief, T.L., Kane, D.A., Lin, C.T., Price, J.W. 3rd, Kang, L., Rabinovitch, P.S., Szeto, H.H., Houmard, J.A., Cortright, R.N., Wasserman, D.H. and Neufer, P.D. (2009) Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J. Clin. Invest. **119**, 573-581

Asayama, K., Nakane, T., Dobashi, K., Kodera, K., Hayashibe, H., Uchida, N. and Nakazawa, S. (2001) Effect of obesity and troglitazone on expression of two glutathione peroxidases: cellular and extracellular types in serum, kidney and adipose tissue. Free Radic. Res. **34**, 337-347

Austin, S. and St-Pierre, J. (2012) PGC1a and mitochondrial metabolism - emerging concepts and relevance in ageing and neurodegenerative disorders. J. Cell Sci. **125**, 4963-4971

Balistreri, C.R., Caruso, C. and Candore, G. (2010) The role of adipose tissue and adipokines in obesity-related inflammatory diseases. Mediators Inflamm., doi:10.1155/2010/802078

Baráth, A., Németh, I., Karg, E., Endreffy, E., Bereczki, C., Gellén, B., Haszon, I. and Túri, S. (2006) Roles of paraoxonase and oxidative stress in adolescents with uraemic, essential or obesity-induced hypertension. Kidney Blood Press. Res. **29**, 144-151

Bastaki, M., Huen, K., Manzanillo, P., Chande, N., Chen, C., Balmes, J.R., Tager, I.B. and Holland, N. (2006) Genotype-activity relationship for Mn-superoxide dismutase, glutathione peroxidases 1 and catalase in humans. Pharmacogenet. Genomics **16**, 279-286

Berhouma, R., Kouidhi, S., Ammar, M., Abid, H., Ennafaa, H. and Benammar-Elgaaied, A. (2013) Correlation of peroxisome proliferator-activated receptor (PPAR-γ) mRNA expression with Pro12Ala polymorphism in obesity. Biochem. Genet. **51**, 256-263

Bondia-Pons, I., Ryan, L. and Martinez, J.A. (2012) Oxidative stress and inflammation interactions in human obesity. J. Physiol. Biochem. **68**, 701-711

Caple, F., Williams, E.A., Spiers, A., Tyson, J., Burtle, B., Daly, A.K., Mathers, J.C. and Hesketh, J.E. (2010) Inter- individual variation in DNA damage and base excision repair in young, healthy nonsmokers: effects of dietary supplementation and genotype. Br. J. Nutr. **103**, 1585-1593

Chartoumpekis, D.V. and Kensler, T.W. (2013) New player on an old field; the Keap1/Nrf2 pathway as a target for treatment of type 2 diabetes and metabolic syndrome. Curr. Diabetes Rev. **9**, 137-145

Chartoumpekis, D.V., Ziros, P.G., Sykiotis, G.P., Zaravinos, A., Psyrogiannis, A.I., Kyriazopoulou, V.E., Spandidos, D.A. and Habeos, I.G. (2011) Nrf2 activation diminishes during adipocyte differentiation of ST2 cells. Int. J. Mol. Med. **28**, 823-238 (a)

Chartoumpekis, D.V., Ziros, P.G., Psyrogiannis, A.I., Papavassiliou, A.G., Kyriazopoulou, V.E., Sykiotis, G.P. and Habeos, I.G. (2011) Nrf2 represses FGF21 during long-term high-fat diet-induced obesity in mice. Diabetes **60**, 2465-2473 (b)

Cho, H.Y. (2013) Genomic Structure and Variation of Nuclear Factor (Erythroid-Derived 2)-Like 2. Oxid. Med. Cell. Longev., doi:10.1155/2013/286524

Codoñer-Franch, P., Tavárez-Alonso, S., Murria-Estal, R., Megías-Vericat, J., Tortajada-Girbés, M. and Alonso-Iglesias, E. (2011) Nitric oxide production is increased in severely obese children and related to markers of oxidative stress and inflammation. Atherosclerosis **2215**, 475-480

Cominetti, C., de Bortoli, M.C., Purgatto, E., Ong, T.P., Moreno, F.S., Garrido, A.B. Jr and Cozzolino, S.M. (2011) Associations between glutathione peroxidase-1 Pro198Leu polymorphism, selenium status, and DNA damage levels in obese women after consumption of Brazil nuts. Nutrition **27**, 891-896

Costa, L.G., Giordano, G. and Furlong, C.E. (2011) Pharmacological and dietary modulators of paraoxonase 1 (PON1) activity and expression: the hunt goes on. Biochem. Pharmacol. **81**, 337-344

Cox, A.G., Winterbourn, C.C. and Hampton, M.B. (2010) Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. Biochem. J. **425**, 313-325

Da Costa, L.A., Badawi, A. and El-Sohemy, A. (2012) Nutrigenetics and modulation of oxidative stress. Ann. Nutr. Metab. **60**, 27-36

Das, S.K., Sharma, N.K., Hasstedt, S.J., Mondal, A.K., Ma, L., Langberg, K.A. and Elbein, S.C. (2011) An integrative genomics approach identifies activation of thioredoxin/thioredoxin reductase-1-mediated oxidative stress defense pathway and inhibition of angiogenesis in obese nondiabetic human subjects. J. Clin. Endocrinol. Metab. **96**, E1308-E1313

DeCoursey, T.E., Ligeti, E. (2005) Regulation and termination of NADPH oxidase activity. Cell. Mol. Life Sci. 62, 2173-2193

Deeb, S.S., Fajas, L., Nemoto, M., Pihlajamäki, J., Mykkänen, L., Kuusisto, J., Laakso, M., Fujimoto, W. and Auwerx, J. (1998) A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. Nat. Genet. **20**, 284-287

El-Koofy, N.M., El-Karaksy, H.M., Mandour, I.M., Anwar, G.M., El-Raziky, M.S. and El-Hennawy, A.M. (2011) Genetic polymorphisms in non-alcoholic fatty liver disease in obese Egyptian children. Saudi J. Gastroenterol. **17**, 265-270

Eriksson, J.W. (2007) Metabolic stress in insulin's target cells leads to ROS accumulation - a hypothetical common pathway causing insulin resistance. FEBS Lett. **581**, 3734-3742

Esterbauer, H., Oberkofler, H., Linnemayr, V., Iglseder, B., Hedegger, M., Wolfsgruber, P., Paulweber, B., Fastner, G., Krempler, F., Patsch, W. (2002) Peroxisome proliferator-activated receptor-gamma coactivator-1 gene locus: associations with obesity indices in middle-aged women. Diabetes **51**, 1281-1286

Fanelli, M., Filippi, E., Sentinelli, F., Romeo, S., Fallarino, M., Buzzetti, R., Leonetti, F. and Baroni, M.G. (2005) The Gly482Ser missense mutation of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 alpha) gene associates with reduced insulin sensitivity in normal and glucose-intolerant obese subjects. Dis. Markers. **21**, 175-180

Finkel, T. (2006) Signal transduction by reactive oxygen species. J. Cell Biol. **194**, 7-15

Forsberg, L., Lyrenás, L., De Faire, U. and Morgenstern, R. (2001) A common functional C-T substitution polymorphism in the promoter region of the human catalase gene influences transcription factor binding, reporter gene transcription and is correlated to blood catalase levels. Free Radic. Biol. Med. **30**, 500-505

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M. and Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. J. Clin. Invest. **114**, 1752-1761

Galbete, C., Toledo, E., Martínez-González, M.A., Martínez, J.A., Guillén-Grima, F. And Marti, A. (2013) Pro12Ala variant of the PPARG2 gene increases body mass index: An updated meta-analysis encompassing 49,092 subjects. Obesity (Silver Spring), doi:10.1002/oby.20150

Garin, M.C., James, R.W., Dussoix, P., Blanché, H., Passá, P., Froguel, P. and Ruiz, J. (1997) Paraoxonase Polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. J. Clin. Invest. **99**, 62-66

Gough, D.R. and Cotter, T.G. (2011) Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis., doi:10.1038/cddis.2011.96

Goyenechea, E., Crujeiras, A.B., Abete, I., Parra, D. and Martínez, J.A. (2008) Enhanced short-term improvement of insulin response to a low-caloric diet in obese carriers the Gly482Ser variant of the PGC-1alpha gene. Diabetes Res. Clin. Pract. **82**, 190-196

Gregor, M.F. and Hotamisligil, G.S. (2011) Inflammatory mechanisms in obesity. Annu. Rev. Immunol. **29**, 415-545

Hamanishi, T., Furuta, H., Kato, H., Doi, A., Tamai, M., Shimomura, H., Sakagashira, S., Nishi, M., Sasaki, H., Sanke, T. and Nanjo, K. (2004) Functional variants in the glutathione peroxidase-1 (GPx-1) gene are associated with increased intima-media thickness of carotid arteries and risk of macrovascular diseases in japanese type 2 diabetic patients. Diabetes **53**, 2455-2460

Hayaishi-Okano, R., Yamasaki, Y., Kajimoto, Y., Sakamoto, K., Ohtoshi, K., Katakami, N., Kawamori, D., Miyatsuka, T., Hatazaki, M., Hazama, Y. and Hori, M. (2003) Association of NAD(P)H oxidase p22 phox gene variation with advanced carotid atherosclerosis in Japanese type 2 diabetes. Diabetes Care **26**, 458-463

Hiroi, M., Nagahara, Y., Miyauchi, R., Misaki, Y., Goda, T., Kasezawa, N., Sasaki, S. and Yamakawa-Kobayashi, K. (2011) The combination of genetic variations in the PRDX3 gene and dietary fat intake contribute to obesity risk. Obesity (Silver Spring) **19**, 882-887

Hou, Y., Xue, P., Bai, Y., Liu, D., Woods, C.G., Yarborough, K., Fu, J., Zhang, Q., Sun, G., Collins, S., Chan, J.Y., Yamamoto, M., Andersen, M.E. and Pi, J. (2012) Nuclear factor erythroid-derived factor 2-related factor 2 regulates transcription of CCAAT/enhancer-binding protein β during adipogenesis. Free Radic. Biol. Med. **52**, 462-472

Houstis, N., Rosen, E.D. and Lander, E.S. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature **440**, 944-948

Huh, J.Y., Kim, Y., Jeong, J., Park, J., Kim, I., Huh, K.H., Kim, Y.S., Woo, H.A., Rhee, S.G., Lee, K.J. and Ha, H. (2012) Peroxiredoxin 3 is a key molecule regulating adipocyte oxidative stress, mitochondrial biogenesis, and adipokine expression. Antioxid. Redox Signal. **16**, 229-243

Itoh, H., Doi, K., Tanaka, T., Fukunaga, Y., Hosoda, K., Inoue, G., Nishimura, H., Yoshimasa, Y., Yamori, Y. and Nakao, K. (1999) Hypertension and insulin resistance: role of peroxisome proliferator-activated receptor gamma. Clin. Exp. Pharmacol. Physiol. **26**, 558-560

Keaney, J.F. Jr, Larson, M.G., Vasan, R.S., Wilson, P.W., Lipinska, I., Corey, D., Massaro, J.M., Sutherland, P., Vita, J.A. and Benjamin, E.J. (2003) Framingham Study: Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler. Thromb. Vasc. Biol. **23**, 434-439

Kim, C., Zheng, T., Lan, Q., Chen, Y., Foss, F., Chen, X., Holford, T., Leaderer, B., Boyle, P., Chanock, S.J., Rothman, N. and Zhang, Y. (2012) Genetic polymorphisms in oxidative stress

pathway genes and modification of BMI and risk of non-Hodgkin lymphoma. Cancer Epidemiol. Biomarkers Prev. **21**, 866-868

Krieger-Brauer, H,I,, Medda, P.K. and Kather, H. (1995) The stimulus-sensitive H_2O_2-generating system present in human fat-cell plasma membranes is multireceptor-linked and under antagonistic control by hormones and cytokines. Biochem. J. **307**, 543-548

Kuzuya, M., Ando, F., Iguchi, A. and Shimokata, H. (2008) Glutathione peroxidase 1 Pro198Leu variant contributes to the metabolic syndrome in men in a large Japanese cohort. Am. J. Clin. Nutr. **87**, 1939-1944

Labrecque, B., Beaudry, D., Mayhue, M., Hallé, C., Bordignon, V., Murphy, B.D. and Palin, M.F. (2009) Molecular characterization and expression analysis of the porcine paraoxonase 3 (PON3) gene. Gene **443**, 110-120

Lee, Y.S., Kim, A.Y., Choi, J.W., Kim, M., Yasue, S., Son, H.J., Masuzaki, H., Park, K.S. and Kim, J.B. (2008) Dysregulation of adipose glutathione peroxidase 3 in obesity contributes to local and systemic oxidative stress. Mol. Endocrinol. **22**, 2176-2189

Lijnen, H.R., Van Hul, M. and Hemmeryckx, B. (2012) Caloric restriction improves coagulation and inflammation profile in obese mice. Thromb. Res. **129**, 74-79

Lundholm, L., Putnik, M., Otsuki, M., Andersson, S., Ohlsson, C., Gustafsson, J.A. and Dahlman-Wright, K. (2008) Effects of estrogen on gene expression profiles in mouse hypothalamus and white adipose tissue: target genes include glutathione peroxidase 3 and cell death-inducing DNA fragmentation factor, alpha-subunit-like effector A. J. Endocrinol. **196**, 547-557

Luo, W., Cao, J., Li, J. and He, W. (2008) Adipose tissue-specific PPARγ deficiency increases resistance to oxidative stress. Exp. Gerontol. **43**, 154-163

Margis, R., Dunand, C., Teixeira, F.K. and Margis-Pinheiro, M. (2008) Glutathione peroxidase family - an evolutionary overview. FEBS J. **275**, 3959-3970

Martínez-Salazar, M.F., Almenares-López, D., García-Jiménez, S., Sánchez-Alemán, M.A., Juantorena-Ugás, A., Ríos, C. and Monroy-Noyola, A. (2011) Relationship between the paraoxonase (PON1) L55M and Q192R polymorphisms and obesity in a Mexican population: a pilot study. Genes Nutr. **6**, 361-368

Marzec, J.M., Christie, J.D., Reddy, S.P., Jedlicka, A.E., Vuong, H., Lanken, P.N., Aplenc, R., Yamamoto, T., Yamamoto, M., Cho, H.Y. and Kleeberger, S.R. (2007) Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury," FASEB J. **21**, 2237-2246

Meher, A.K., Sharma, P.R., Lira, V.A., Yamamoto, M., Kensler, T.W., Yan, Z. and Leitinger, N. (2012) Nrf2 deficiency in myeloid cells is not sufficient to protect mice from high-fat diet-induced adipose tissue inflammation and insulin resistance. Free Radic. Biol. Med. 52, 1708-1715

Montano, M.A., Barrio Lera, J.P., Gottlieb, M.G., Schwanke, C.H., da Rocha, M.I., Manica-Cattani, M.F., dos Santos, G.F. and da Cruz, I.B. (2009) Association between manganese superoxide dismutase (MnSOD) gene polymorphism and elderly obesity. Mol. Cell. Biochem. **328**, 33-40

Montano, M.A., da Cruz, I.B., Duarte, M.M., Krewer Cda, C., da Rocha, M.I., Mânica-Cattani, M.F., Soares, F.A., Rosa, G., Maris, A.F., Battiston, F.G., Trott, A. and Lera, J.P. (2012) Inflammatory cytokines in vitro production are associated with Ala16Val superoxide dismutase gene polymorphism of peripheral blood mononuclear cells. Cytokine **60**, 30-33

Nadif, R., Mintz, M., Jedlicka, A., Bertrand, J.P., Kleeberger, S.R., Kauffmann, F. (2005) Association of CAT polymorphisms with catalase activity and exposure to environmental oxidative stimuli. Free Radic. Res. **39**, 1345-1350

Nakao, C., Ookawara, T., Sato, Y., Kizaki, T., Imazeki, N., Matsubara, O., Haga, S., Suzuki, K., Taniguchi, N. and Ohno, H. (2000) Extracellular superoxide dismutase in tissues from obese (ob/ob) mice. Free Radic. Res. **33**, 229-241

Nitz, I., Ewert, A., Klapper, M. and Döring, F. (2007) Analysis of PGC-1alpha variants Gly482Ser and Thr612Met concerning their PPARgamma2-coactivation function. Biochem. Biophys. Res. Commun. **353**, 481-486

Ochoa, M.C., Razquin, C., Zalba, G., Martínez-González, M.A., Martínez, J.A. and Marti, A. (2008) G allele of the -930A>G polymorphism of the CYBA gene is associated with insulin resistance in obese subjects. J. Physiol. Biochem. **64**, 127-133

Okauchi, Y., Iwahashi, H., Okita, K., Yuan, M., Matsuda, M., Tanaka, T., Miyagawa, J., Funahashi, T., Horikawa, Y., Shimomura, I. and Yamagata, K. (2008) PGC-1alpha Gly482Ser polymorphism is associated with the plasma adiponectin level in type 2 diabetic men. Endocr. J. **55**, 991-997

Pi, J., Leung, L., Xue, P., Wang, W., Hou, Y., Liu, D., Yehuda-Shnaidman, E., Lee, C., Lau, J., Kurtz, T.W. and Chan, J.Y. (2010) Deficiency in the nuclear factor E2-related factor-2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity. J. Biol. Chem. **285**, 9292-9300

Rupérez, A.I., Olza, J., Gil-Campos, M., Leis, R., Mesa, M.D., Tojo, R., Cañete, R., Gil, A. and Aguilera, C.M. (2013) Are Catalase -844A/G Polymorphism and Activity Associated with Childhood Obesity? Antioxid. Redox Signal., doi:10.1089/ars.2013.5386 (a)

Rupérez, A.I., López-Guarnido, O., Gil, F., Olza, J., Gil-Campos, M., Leis, R., Tojo, R., Cañete, R., Gil, A. and Aguilera, C.M.: (2013) Paraoxonase 1 activities and genetic variation in childhood obesity. Br. J. Nutr. **21**, 1-9 (b)

San José, G., Moreno, M.U., Olivan, S., Beloqui, O., Fortuno, A., Díez, J. and Zalba, G. (2004) Functional effect of the p22phox -930A/G polymorphism on p22phox expression and NADPH oxidase activity in hypertension. Hypertension **44**, 163-169

Schreiber, R., Ferreira-Sae, M.C., Tucunduva, A.C., Mill, J.G., Costa, F.O., Krieger, J.E., Franchini, K.G., Pereira, A.C., Nadruz, W. Jr. (2012) CYBA C242T polymorphism is associated with obesity and diabetes mellitus in Brazilian hypertensive patients. Diabet. Med. **29**, e55-61

Shamseddeen, H., Getty, J.Z., Hamdallah, I.N. and Ali, M.R. (2011) Epidemiology and economic impact of obesity and type 2 diabetes. Surg. Clin. North Am. **91**, 1163-1172

Shimoda-Matsubayashi, S., Matsumine, H., Kobayashi, T. and Nakagawa-Hattori, Y. (1996) Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. Biochem. Biophys. Res. Commun. **226**, 561-565

Shin, M.J. and Park, E. (2006) Contribution of insulin resistance to reduced antioxidant enzymes and vitamins in nonobese Korean children. Clin. Chim. Acta **365**, 200-205

Shin, S., Wakabayashi, J., Yates, M.S., Wakabayashi, N., Dolan, P.M., Aja, S., Liby, K.T., Sporn, M.B., Yamamoto, M. and Kensler, T.W. (2009) Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide. Eur. J. Pharmacol. **620**, 138-144

Shuvalova, Y.A., Kaminnyi, A.I., Meshkov, A.N. and Kukharchuk, V.V. (2010) Pro198Leu polymorphism of GPx-1 gene and activity of erythrocytic glutathione peroxidase and lipid peroxidation products. Bull. Exp. Biol. Med. **149**, 743-745

Sutton, A., Khoury, H., Prip-Buus, C., Cepanec, C., Pessayre, D. and Degoul, F. (2003) The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. Pharmacogenetics **13**, 145-157

Trayhurn, P. (2013) Hypoxia and adipose tissue function and dysfunction in obesity. Physiol. Rev. **93**, 1-21

Trayhurn, P. and Wood, I.S. (2004) Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br. J. Nutr. **92**, 347-355

Valle, I., Alvarez-Barrientos, A., Arza, E., Lamas, S. and Monsalve, M. (2005) PGC-1a regulates the mitochondrial antioxidant defense system in vascular endothelial cells. Cardiovasc. Res. **66**, 562-573

Veiga, L., Silva-Nunes, J., Melão, A., Oliveira, A., Duarte, L. and Brito, M. (2011) Q192R polymorphism of the paraoxonase-1 gene as a risk factor for obesity in Portuguese women. Eur. J. Endocrinol. **164**, 213-218

Vidal-Puig, A.J., Considine, R.V., Jimenez-Liñan, M., Werman, A., Pories, W.J., Caro, J.F. and Flier, J.S. (1997) Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. J. Clin. Invest. **99**, 2416-2422

Vomhof-DeKrey, E.E. and Picklo, M.J. (2012) NAD(P)H:quinone oxidoreductase 1 activity reduces hypertrophy in 3T3-L1 adipocytes. Free Radic. Biol. Med. **53**, 690-700

Wang, Z., Li, Y., Wang, B., He, Y., Wang, Y., Xi, H., Li, Y., Wang, Y., Zhu, D., Jin, J., Huang, W. and Jin, L. (2010) A haplotype of the catalase gene confers an increased risk of essential hypertension in Chinese Han. Hum. Mutat. **31**, 272-278

Weng, S.W., Lin, T.K., Wang, P.W., Chen, I.Y., Lee, H.C., Chen, S.D., Chuang, Y.C. and Liou, C.W. (2010) Gly482Ser polymorphism in the peroxisome proliferator-activated receptor gamma coactivator-1alpha gene is associated with oxidative stress and abdominal obesity. Metabolism **59**, 581-586

Zelko, I.N., Mariani, T.J. and Folz, R.J. (2002) Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), MN-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic. Biol. Med. **33**, 337-349

Zielekiak, A., Wójcik, M. and Woźniak, L.A. (2008) Structure and physiological functions of the human peroxisome proliferator-activated receptor γ. Arch. Immunol. Ther. Exp. **56**, 331-345

Chapter 2

General Results

RESULTS

General characteristics of the population under study

Table 2.1 shows the anthropometric and blood pressure values of the obese and normal-weight children. Obese children showed significantly higher values of weight, BMI, BMI *z*-score and WC. SBP and DPB were also significantly increased with obesity in these children.

Table 2.1. Anthropometry and blood pressure in normal-weight and obese children.

Variable	Normal-weight	Obese	*P*
Sex (male/female)	110/81	104/89	
Age (y)	8.9±0.1	8.7±0.1	0.127
Weight (kg)	29.2±0.4	51.3±0.9	< 0.001
BMI (kg/m^2)	16.60±0.11	26.94±0.26	< 0.001
BMI *z*-score	-0.22±0.04	3.57±0.11	< 0.001
WC (cm)	58.3±0.5	81.3±0.9	< 0.001
SBP (mmHg)	95±1	110±1	< 0.001
DBP (mmHg)	58±1	69±1	< 0.001

BMI: body mass index; DBP: diastolic blood pressure; SBP: systolic blood pressure; WC: waist circumference.

Regarding insulin resistance biomarkers (Table 2.2), fasting insulin and HOMA-IR were significantly higher and QUICKI was significantly lower in obese children, whereas fasting glucose values did not differ between obese and normal-weight children.

Table 2.2. Insulin resistance biomarkers in normal-weight and obese children.

Variable	Normal-weight	Obese	*P*
Glucose (mg/dL)	83±1	84±1	0.846
Insulin (mU/L)	5.2±0.2	10.3±0.5	< 0.001
QUICKI	0.390±0.002	0.351±0.002	< 0.001
HOMA-IR	1.08±0.04	2.14±0.11	< 0.001

HOMA-IR: homeostasis model assessment for insulin resistance; QUICKI: quantitative insulin sensitivity check index.

Liver transaminases ALT and GGT were significantly higher in obese children, whereas AST was lower (Table 2.3).

Table 2.3. Hepatic enzymes in normal-weight and obese children.

Variable	Normal-weight	Obese	*P*
AST (U/L)	25.4±0.5	22.9±0.5	<0.001
ALT (U/L)	17.4±0.7	22.2±0.7	<0.001
GGT (U/L)	9.1±0.3	11.9±0.4	<0.001

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamil transferase.

Plasma lipid metabolism biomarkers (Figure 2.1 A) also showed differences between groups. Total cholesterol, HDL-C and ApoA-I were significantly lower in obese children. On the contrary, TG, ApoB and FABP-4 were higher in the obese compared to normal-weight children. LDL-C was not significantly different between groups.

Fasting plasma concentrations of resistin and leptin were significantly higher in obese than in normal-weight subjects, by contrast decreased levels of adiponectin were found in obese children (Figure 2.1 B). Inflammation and endothelial damage biomarkers also showed differences between the groups. Regarding endothelial damage biomarkers, MPO, active PAI-1 and total PAI-1 were significantly higher in the obese group compared to the normal-weight group, whereas MMP-9 was not (Figure 2.1 C). The inflammation markers hsCRP, IL-6, IL-8 and TNF-α were significantly higher in the obese group compared to the normal-weight group, whereas MCP-1 concentration did not show any difference between groups (Figure 2.1 D).

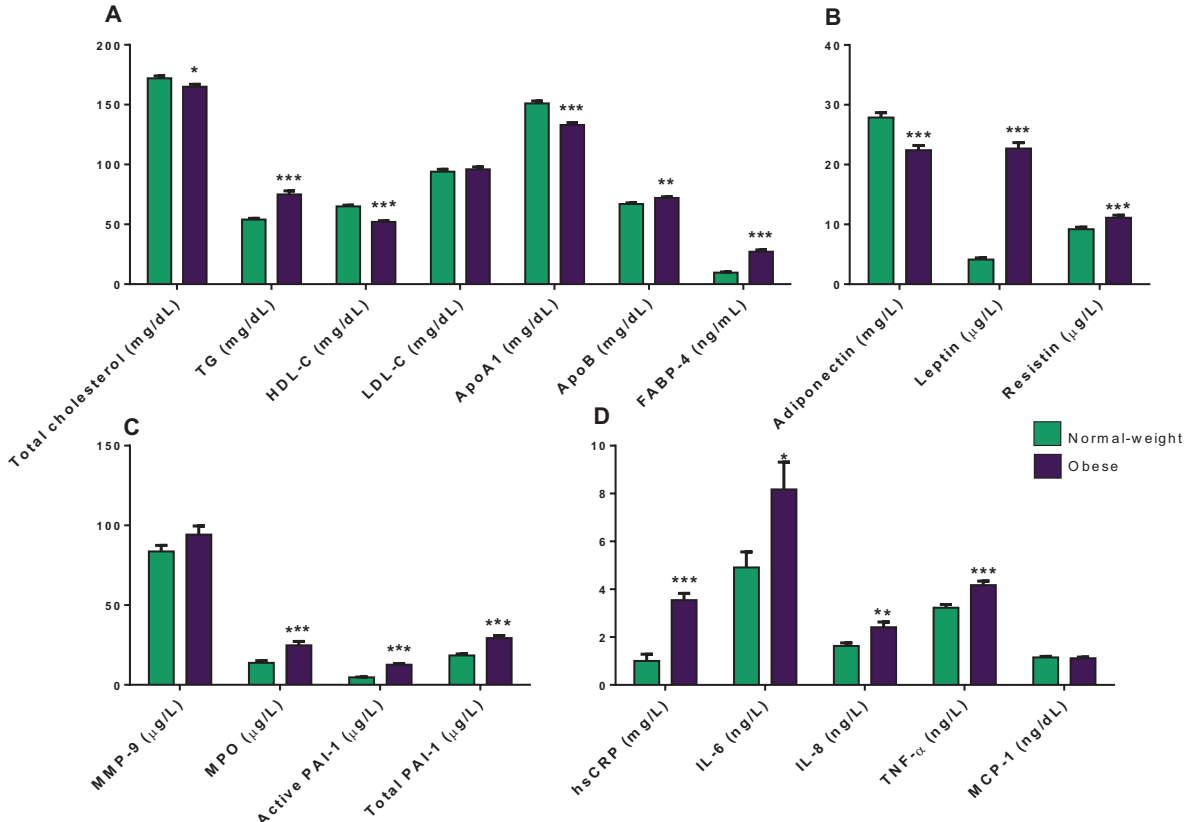

Figure 2.1. *A: Lipid metabolism biomarkers; B: Adipokines; C: Endothelial damage biomarkers; D: Inflammation biomarkers in obese and normal-weight children. Y axis: units indicated in the X-axis. X axis: Parameters (units). ALT: alanine aminotransferase; ApoA1: apolipoprotein A-I; ApoB: apolipoprotein B; AST: aspartate aminotransferase; FABP-4: fatty acid-binding protein 4; GGT: gamma-glutamil transferase; HDL-C: high-density lipoprotein cholesterol; hsCRP: high sensitivity C-reactive protein; IL-6: interleukin 6; IL-8: interleukin 8; LDL-C: low-density lipoprotein cholesterol; MCP-1: monocyte chemoattractant protein-1; MMP-9: Matrix metallopeptidase 9; MPO: myeloperoxidase; PAI-1: plasminogen activator inhibitor 1; TG: triacylclycerols. TNF-α: tumor necrosis factor alpha. *p<0.05; **p<0.01; **p<0.001.*

Figure 2.2 shows the difference in oxidative stress-related markers between obese and normal-weight children. CAT activity in erythrocytes was found to be significantly lower in obese children, but no differences were observed between erythrocyte enzymatic activities of GR, GPX, SOD or PON1 between obese and normal-weight children (Figure 2.2 A and B). Regarding plasmatic markers of oxidative stress, ox-LDL was not significantly different between the groups, whereas TAC was significantly lower in obese children (Figure 2.2 C). The analyzed vitamins α-tocopherol and β-carotene were significantly lower in obese children, with no differences found in retinol between groups (Figure 2.2 D and E).

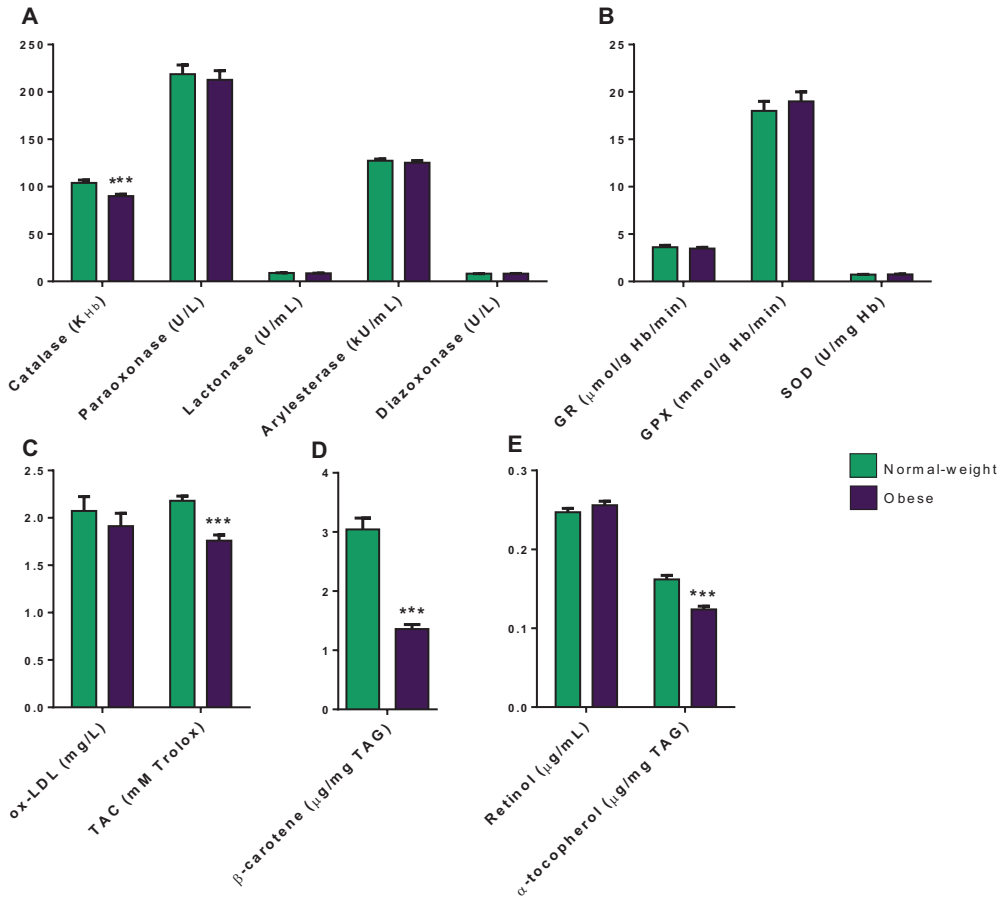

Figure 2.2. *A and B: antioxidant enzyme's activities; C: Oxidative stress markers in plasma; D and E: Vitamins in obese and normal-weight children. Y axis: units indicated in the X-axis. X axis: Parameters (units). GPX: glutathione peroxidase; GR: glutathione reductase; ox-LDL: oxidized LDL; SOD: superoxide dismutase; TAC: total antioxidant capacity. ***p<0.001.*

Genotyping results

Genotype frequency

The genotyping results from all the selected SNPs from antioxidant defense system-related genes are shown in the "Table of genotyped SNPs" (Appendix). From these data it can be observed that the retrieved frequencies are similar to those from HapMap in Caucasians, which indicates the accurateness of the genotyping.

Association with obesity

The association analysis showed that 19 SNPs from the total of 492 analyzed were associated with childhood obesity (Table 2.4). The same results can be observed in a different way in Figure 2.3, which shows the positive associations (OR>1) which increase obesity risk in red, and the negative associations (OR<1) which confer protection against obesity in green.

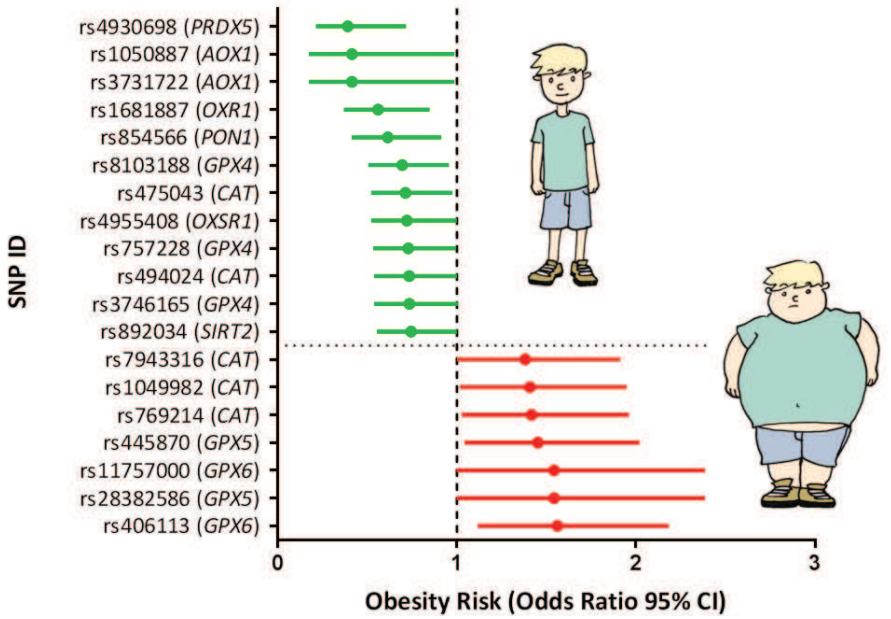

Figure 2.3. *Graph representing the Odds Ratio of SNPs significantly associated with obesity. Green lines indicate negative (protective) associations, whereas red lines indicate positive (risk) associations. CI: confidence interval; SNP ID: official code of the SNP (gene).*

Table 2.4. SNPs significantly associated with childhood obesity.

SNP	Gene	Chr	Chr Position	Risk allele	MAF Ref	MAF	Function	OR	*P**
rs4930698	*PRDX5*	11	63841639	C	0.114	0.080	5'-UTR	0.391	0.002
rs1681887	*OXR1*	8	107743689	G	0.167	0.166	tag SNP	0.561	0.006
rs406113	*GPX6*	6	28591461	C	0.285	0.352	missense	1.562	0.009
rs11757000	*GPX6*	6	28592848	G	0.175	0.147	3'-UTR	1.544	0.051
rs854566	*PON1*	7	94786685	A	0.150	0.183	tag SNP	0.615	0.016
rs8103188	*GPX4*	19	1054465	G	0.433	0.444	5'-UTR	0.696	0.026
rs757228	*GPX4*	19	1052992	G	0.475	0.497	5'-UTR	0.729	0.048
rs3746165	*GPX4*	19	1053211	G	0.475	0.499	5'-UTR	0.736	0.055
rs445870	*GPX5*	6	28602306	G	0.354	0.330	tag SNP	1.452	0.027
rs28382586	*GPX5*	6	28600461	C	0.136	0.147	5'-UTR	1.544	0.051
rs769214	*CAT*	11	34416293	G	0.299	0.346	5'-UTR	1.418	0.035
rs1049982	*CAT*	11	34417117	A	0.292	0.347	5'-UTR	1.408	0.039
rs7943316	*CAT*	11	34417048	T	0.275	0.349	5'-UTR	1.382	0.050
rs475043	*CAT*	11	34450377	G	0.458	0.342	3'-UTR	0.714	0.035
rs494024	*CAT*	11	34421324	A	0.412	0.345	tag SNP	0.734	0.051
rs3731722	*AOX1*	2	201242634	G	0.033	0.036	missense	0.415	0.047
rs1050887	*AOX1*	2	201244175	A	0.050	0.036	3'-UTR	0.415	0.047
rs4955408	*OXSR1*	3	38227410	A	0.321	0.295	tag SNP	0.722	0.050
rs892034	*SIRT2*	19	44060838	A	0.466	0.458	5'-UTR	0.745	0.050

**P statistical significance of the additive model (adjusted by sex and age). AOX1: aldehyde oxidase 1; CAT: catalase; Chr: chromosome; GPX4: glutathione peroxidase 4; GPX5: glutathione peroxidase 5; GPX6: glutathione peroxidase 6; MAF: minor allele frequency; MAF Ref: MAF from HapMap for Caucasian population; OR: odds ratio; OXR1: oxidation resistance 1; OXSR1: oxidative stress responsive 1;; PON1: paraoxonase 1; PRDX5: peroxiredoxin 5; SIRT2: sirtuin 2.*

Association of gene-specific SNPs with obesity related phenotypes and other biomarkers

Paraoxonase 1 (PON1)

The results from the association analysis of *PON1* SNPs are included in Chapter 3 as a publication part of this thesis. Nevertheless, the main significant findings regarding its association with phenotypes are summarized in Table 2.5, were it can be observed that the presence of the minor allele of rs854566 is associated with lower BMI, WC, weight and PON1 paraoxonase activity. In addition, this SNP is associated with higher PON1 lactonase, diazoxonase and arylesterase activities. The association of this genetic variant and the different PON1 activities remained significant after the additional adjustment by BMI, indicating an independent effect on the enzyme's activities and obesity.

Table 2.5. Significant associations of rs854566 SNP from *PON1* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.319	-2.426	-0.212	0.02007
WC (cm)	-0.536	-0.934	-0.137	0.00878
Weight (kg)	-3.031	-5.579	-0.484	0.02027
Paraoxonase (U/l)	-56.100	-95.160	-17.040	0.00563*
Arylesterase (kU/ml)	14.880	7.173	22.590	0.00023*
Lactonase (U/ml)	0.757	0.247	1.266	0.00425*
Diazoxonase (U/l)	1.271	0.718	1.823	0.00001*

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; WC: waist circumference.*

Catalase (CAT)

The results from the association of *CAT* SNPs with obesity and its phenotypes are included as publication in this thesis, in Chapter 4. The main significant results are shown in Tables 2.6 to 2.10. We observed that the previously known SNP rs769214 was associated with higher BMI, BMI *z*-score, weight, ApoB and FABP-4, all markers of obesity. In the same way, SNPs rs7943316 and rs1049982, in LD with the mentioned SNP, showed very similar associations with the same phenotypes.

Table 2.6. Significant associations of rs769214 SNP from *CAT* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	1.217	0.301	2.132	0.00958
BMI *z*-score	0.429	0.098	0.759	0.01144
Weight (kg)	2.935	0.829	5.040	0.00661
ApoB (mg/dl)	2.969	0.090	5.848	0.04405
FABP-4 (ng/ml)	4.835	1.088	8.582	0.01332

ApoB: apolipoprotein B; BMI: body mass index; FABP-4: fatty acid-binding protein 4; WC: waist circumference.

Table 2.7. Significant associations of rs7943316 SNP from *CAT* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	1.137	0.219	2.055	0.01570
BMI *z*-score	0.407	0.075	0.738	0.01667
Weight (kg)	2.761	0.650	4.872	0.01078
FABP-4 (ng/ml)	4.525	0.751	8.299	0.02114

BMI: body mass index; FABP-4: fatty acid-binding protein 4; WC: waist circumference.

Table 2.8. Significant associations of rs1049982 SNP from *CAT* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	1.200	0.283	2.117	0.01075
BMI *z*-score	0.424	0.093	0.755	0.01255
Weight (kg)	2.919	0.808	5.030	0.00704
ApoB (mg/dl)	2.969	0.090	5.848	0.04405
FABP-4 (ng/ml)	4.835	1.088	8.582	0.01332

ApoB: apolipoprotein B; BMI: body mass index; FABP-4: fatty acid-binding protein 4; WC: waist circumference.

As a novel finding, we observed the negative association of SNPs rs475043 and rs494024 with obesity, and their association with lower weight and higher HDL-C. SNP rs494024 was also associated with higher ApoA-I levels.

Table 2.9. Significant associations of rs475043 SNP from *CAT* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
Weight (kg)	-2.225	-4.266	-0.183	0.03336
HDL-C (mg/dl)	2.308	0.027	4.589	0.04808

HDL-C: high-density lipoprotein cholesterol.

Table 2.10. Significant associations of rs494024 SNP from *CAT* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
Weight (kg)	-2.147	-4.181	-0.113	0.03930
ApoA-I (mg/dl)	4.398	0.242	8.555	0.03882
HDL-C (mg/dl)	2.352	0.081	4.623	0.04310

ApoA-I: apolipoprotein A-I; HDL-C: high-density lipoprotein cholesterol.

Glutathione peroxidases (GPXs)

The GPXs' SNPs included in this thesis belonged to *GPX1* to *GPX7*. From all the analyzed SNPs, we found associations in SNPs of *GPX4*, *GPX5* and *GPX6*.

In the *GPX4* gene we found the SNPs rs757228, rs3746165 and rs8103188 in the 5'-UTR to be associated with lower obesity risk (Table 2.4). When we observed their association with obesity phenotypes and the rest of the analyzed biomarkers we confirmed the protection against obesity (Table 2.11-2.13). The three SNPs were significantly associated with lower BMI, BMI *z*-score, WC and weight. Moreover, the protective allele of each of these SNPs was associated with lower DBP, SBP and leptin; and with a higher TAC. In the case of rs8103188 the minor allele was also associated with higher HDL-C, lower TG and higher α-tocopherol. None of these SNPs was found to be associated with GPX activity in erythrocytes. Like ways, SNP rs757228 was not associated with GPX activity in a previous study by Zanon-Moreno *et al*. (2013).

Table 2.11. Significant associations of rs757228 SNP from *GPX4* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.190	-2.075	-0.305	0.00880
BMI *z*-score	-0.430	-0.749	-0.111	0.00870
WC (cm)	-2.902	-5.254	-0.550	0.01615
Weight (kg)	-3.060	-5.093	-1.027	0.00339
DBP (mmHg)	-2.248	-4.187	-0.309	0.02373
SBP (mmHg)	-3.757	-6.073	-1.442	0.00161
Leptin (μg/l)	-2.214	-4.321	-0.106	0.04027
TAC (mM Trolox)	0.177	0.048	0.306	0.00755*

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; DBP: diastolic blood pressure; SBP: systolic blood pressure; TAC: total antioxidant capacity; WC: waist circumference.*

Table 2.12. Significant associations of rs3746165 SNP from *GPX4* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.181	-2.069	-0.293	0.00956
BMI *z*-score	-0.426	-0.747	-0.106	0.00951
WC (cm)	-2.889	-5.249	-0.529	0.01696
Weight (kg)	-3.059	-5.098	-1.019	0.00350
DBP (mmHg)	-2.303	-4.248	-0.359	0.02087
SBP (mmHg)	-3.782	-6.104	-1.459	0.00155
Leptin (μg/l)	-2.194	-4.308	-0.079	0.04277
TAC (mM Trolox)	0.180	0.050	0.309	0.00680*

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; DBP: diastolic blood pressure; SBP: systolic blood pressure; TAC: total antioxidant capacity; WC: waist circumference.*

Table 2.13. Significant associations of rs8103188 SNP from *GPX4* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.352	-2.250	-0.453	0.00340
BMI *z*-score	-0.519	-0.843	-0.195	0.00181
WC (cm)	-3.198	-5.593	-0.802	0.00931
Weight (kg)	-3.060	-5.129	-0.991	0.00399
DBP (mmHg)	-3.093	-5.046	-1.140	0.00207
SBP (mmHg)	-3.608	-5.956	-1.260	0.00280
HDL-C (mg/dl)	2.681	0.370	4.993	0.02358
TG (mg/dl)	-5.226	-10.120	-0.336	0.03691
Leptin (μg/l)	-3.413	-5.541	-1.284	0.00182
TAC (mM Trolox)	0.140	0.008	0.271	0.03785
α-tocopherol (μg/mg TG)	10.450	0.388	20.510	0.04256

BMI: body mass index; DBP: diastolic blood pressure; HDL-C: high-density lipoprotein cholesterol; SBP: systolic blood pressure; TAC: total antioxidant capacity; TG: triacylclycerols; WC: waist circumference.

Two SNPs from *GPX5* were positively associated with obesity in children. The presence of the risk allele of SNP rs28382586 was associated with an increase in BMI *z*-score and WC (Table 2.14). In the same way, SNP rs445870 was associated with higher BMI, BMI *z*-score and leptin (Table 2.15).

Table 2.14. Significant associations of rs28382586 SNP from *GPX5* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI *z*-score	0.469	0.026	0.913	0.03891
WC (cm)	3.601	0.361	6.841	0.03008

WC: waist circumference

Table 2.15. Significant associations of rs445870 SNP from *GPX5* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	1.005	0.071	1.940	0.03570
BMI *z*-score	0.412	0.075	0.748	0.01700
Leptin (μg/l)	2.561	0.350	4.772	0.02380

BMI: body mass index.

Concerning GPX6, two SNPs were found in this gene to be associated with a higher childhood obesity risk, rs406113 and rs11757000. SNP rs406113 was found to be associated with higher BMI, BMI *z*-score and leptin (Table 2.16), whereas rs11757000 was observed to be associated with higher BMI *z*-score and WC (Table 2.17).

Table 2.16. Significant associations of rs406113 SNP from *GPX6* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	1.197	0.257	2.137	0.01304
BMI *z*-score	0.475	0.137	0.814	0.00624
Leptin (μg/l)	2.558	0.328	4.789	0.02519

BMI: body mass index.

Table 2.17. Significant associations of rs11757000 SNP from *GPX6* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI *z*-score	0.469	0.026	0.913	0.03891
WC (cm)	3.601	0.361	6.841	0.03008

BMI: body mass index; WC: waist circumference.

Aldehyde oxidase 1 (AOX1)

AOX1 is a cytosolic drug-metabolizing enzyme localized in many tissues (Garattini *et al.* 2009). Its main function is to oxidize a broad range of aldehydes and drug metabolites. Two SNPs in the aldehyde oxidase 1 gene (*AOX1*) showed association with lower obesity in children. Confirming the observed association with the disease, SNPs rs3731722 and rs1050887 were also associated with lower BMI, WC, weight and leptin, all markers of obesity (Tables 2.18 and 2.19). Surprisingly, the association between the presence of these SNPs and WC remained significant after additional adjustment by BMI, indicating an independent effect of the SNPs on WC. Hartmann *et al.* described how the SNP rs3731722 (also called H1297R) has an impact increasing the metabolizing efficiency of AOX1 (Hartmann *et al*. 2012).

Table 2.18. Significant associations of rs3731722 SNP from *AOX1* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-2.632	-4.937	-0.327	0.02584
WC (cm)	-9.478	-15.420	-3.540	0.00191*
Weight (kg)	-6.755	-12.050	-1.459	0.01288
Leptin (µg/l)	-6.349	-11.800	-0.900	0.02297

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; WC: waist circumference.*

Table 2.19. Significant associations of rs1050887 SNP from *AOX1* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-2.632	-4.937	-0.327	0.02584
WC (cm)	-9.478	-15.420	-3.540	0.00191*
Weight (kg)	-6.755	-12.050	-1.459	0.01288
Leptin (µg/l)	-6.349	-11.800	-0.900	0.02297

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; WC: waist circumference.*

Peroxiredoxin 5 (PRDX5)

PRDX5 is located in peroxisomes, mitochondria and cytosol (Wood *et al*. 2003). SNP rs4930698 from the *PRDX5* gene was observed to be negatively associated with obesity, protecting against it in children. In the same way, it showed a negative association with BMI, WC and weight (Table 2.20). Moreover, the presence of the protective allele was associated with lower values of MPO and hsCRP, markers of endothelial damage and inflammation.

Table 2.20. Significant associations of rs4930698 SNP from *PRDX5* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.890	-3.491	-0.288	0.02131
WC (cm)	-5.161	-9.479	-0.844	0.01971
Weight (kg)	-4.823	-8.503	-1.143	0.01062
MPO (µg/l)	-8.017	-15.020	-1.015	0.02546
hsPCR (mg/l)	-1.251	-2.373	-0.128	0.02971

BMI: body mass index; hsCRP: high-sensitivity C-reactive protein; MPO: myeloperoxidase; WC: waist circumference.

Oxidation resistance 1 (OXR1)

OXR1 is involved in the protection against cellular oxidative stress. In this study, the SNP rs1681887 from the intron region of the oxidation resistance 1 gene (*OXR1*) was found to be negatively associated with childhood obesity. The results of the significant found associations with the analyzed biomarkers are shown in Table 2.21. In this table we can observe how the association is confirmed by the presence of lower BMI, BMI *z*-score, WC and weight linked to the presence of the protective allele. Surprisingly, we also observed that the minor allele was associated with lower SBP, HOMA-IR and insulin values; and with higher QUICKI, β-carotene, α-tocopherol and GPX and GR activities in erythrocytes. In fact, the associations between rs1681887 and GPX and GR activities remained significant after an additional adjustment by BMI.

Table 2.21. Significant associations of rs1681887 SNP from *OXR1* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.387	-2.535	-0.240	0.01833
BMI *z*-score	-0.488	-0.903	-0.074	0.02140
WC (cm)	-3.348	-6.388	-0.307	0.03163
Weight (kg)	-2.976	-5.620	-0.332	0.02803
SBP (mmHg)	-3.126	-6.139	-0.112	0.04286
HOMA-IR	-0.277	-0.523	-0.032	0.02744
Insulin (mU/l)	-1.289	-2.351	-0.227	0.01790
QUICKI	0.008	0.001	0.016	0.02411
GPX (mol/g Hb/min)	0.003	0.000	0.007	0.04487*
GR (μmol/g Hb/min)	0.592	0.137	1.047	0.01119*
β-carotene (μg/mg TG)	0.526	0.098	0.955	0.01666
α-tocopherol (μg/ mg TG)	14.060	1.226	26.890	0.03246

**Associations remain significant after an additional adjustment by BMI. BMI: body mass index; GPX: glutathione peroxidase; GR: glutathione reductase; HOMA-IR: homeostasis model assessment for insulin resistance; SBP: systolic blood pressure; TG: triacylclycerols; QUICKI: quantitative insulin sensitivity check index; WC: waist circumference*.

Oxidative stress responsive 1 (OXSR1)

OXSR1 is a Serine-Threonine protein kinase which responds to environmental stresses such as osmotic stress (Chen *et al*. 2004). The SNP rs4955408 in the oxidative stress responsive 1 gene (*OXSR1*) was also found to be negatively associated with obesity. As in the previous variants, this SNP was also associated with lower BMI, BMI *z*-score, WC, weight and leptin, thus confirming the observed association with childhood obesity (Table 2.22). In addition, we observed this SNP to be associated with protection against insulin resistance by lower HOMA-IR and insulin values and higher QUICKI. In addition, the presence of the protective allele was associated with higher β-carotene, α-tocopherol and TAC values, indicating lower oxidative stress risk; and with lower active PAI-1 and MPO, markers of endothelial damage.

Table 2.22. Significant associations of rs4955408 SNP from *OXSR1* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
BMI (kg/m^2)	-1.251	-2.171	-0.332	0.00799
BMI *z*-score	-0.433	-0.765	-0.101	0.01104
WC (cm)	-3.607	-6.072	-1.142	0.00439
Weight (kg)	-2.610	-4.730	-0.489	0.01636
HOMA-IR	-0.283	-0.479	-0.087	0.00494
Insulin (mU/l)	-1.302	-2.149	-0.454	0.00281
Leptin (µg/l)	-2.684	-4.867	-0.502	0.01643
QUICKI	0.007	0.002	0.013	0.01281
TAC (mM Trolox)	0.155	0.020	0.290	0.02464
β-carotene (µg/mg TG)	0.362	0.017	0.708	0.04063
α-tocopherol (µg/mg TG)	11.580	1.268	21.890	0.02839
a-PAI-1 (µg/l)	-1.412	-2.788	-0.036	0.04513
MPO (µg/l)	-4.283	-8.324	-0.242	0.03850

a-PAI-1: active plasminogen activator inhibitor 1; BMI: body mass index; HOMA-IR: homeostasis model assessment for insulin resistance; MPO: myeloperoxidase; TAC: total antioxidant capacity; TG: triacylclycerols; QUICKI: quantitative insulin sensitivity check index; WC: waist circumference.

Sirtuin 2 (SIRT2)

SIRT2 is a class I protein of the sirtuin family localized mainly in the cytoplasm. It is the most abundant sirtuin in adipocytes and it mediates an inhibitory effect on adipocyte differentiation (Jing *et al.* 2007). The SNP rs892034 in the promoter region of the *SIRT2* gene was observed to be associated with lower childhood obesity risk. After analyzing its effect on the studied biomarkers we observed that its association was not confirmed by lower BMI or other obesity related phenotypes (Table 2.23). However, the presence of the minor allele was associated with higher HDL-C and α-tocopherol; and with lower IL-8 and MMP-9, markers of inflammation and endothelial damage, respectively. Additional studies could be useful to elucidate the functional effect of this SNP. A higher *SIRT2* expression in adipose tissue might inhibit differentiation, thus providing a possible explanation of this SNP's function protecting against obesity.

Table 2.23. Significant associations of rs892034 SNP from *SIRT2* gene with obesity related phenotypes.

Phenotype	BETA	Lower 95%CI	Upper 95%CI	P
HDL-C (mg/dL)	2.461	0.299	4.623	0.02631
IL-8 (ng/L)	-0.381	-0.735	-0.027	0.03568
MMP-9 (μg/L)	-12.300	-21.540	-3.053	0.00952
α-tocopherol (μg/ mg TG)	11.620	2.266	20.960	0.01539

HDL-C: high-density lipoprotein cholesterol; IL-8: interleukin 8; MMP-9: matrix metalloproteinase 9; TG: triacylclycerols.

Chapter 3

Paraoxonase 1 Activity and Genetic Variation in Childhood Obesity

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Paraoxonase 1 activities and genetic variation in childhood obesity

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Abstract

Changes in paraoxonase 1 (PON1) activities have been observed in a variety of diseases involving oxidative stress, such as CVD. However, its role in obesity has not been fully established. In the present study, we aimed (1) to genotype sixteen *PON1* SNP, (2) to measure serum PON1 activities and (3) to correlate these findings with the incidence of childhood obesity and related traits. We conducted a case–control study of 189 normal-weight and 179 obese prepubertal children, and we measured four different PON1 activities: lactonase; paraoxonase; arylesterase; diazoxonase. Although none of these activities was significantly different between the obese and normal-weight children, lactonase activity was found to be positively correlated with HDL-cholesterol and ApoA1 levels and negatively correlated with myeloperoxidase and fatty acid-binding protein 4 levels. Among the sixteen genotyped *PON1* SNP, only the intronic SNP rs854566 exhibited a significant association with obesity (OR 0·61, 95 % CI 0·41, 0·91; $P=0·016$). This genetic variant was also associated with increased diazoxonase, lactonase and arylesterase activities and decreased paraoxonase activity. Other genetic variants exhibited different association patterns with serum activities based on their location within the *PON1* gene, and SNP that were located within the promoter were strongly associated with lactonase, arylesterase and diazoxonase activities. The functional variant Q192R exhibited the greatest effect on paraoxonase activity ($P=5·88\times10^{-42}$). In conclusion, SNP rs854566 was negatively associated with childhood obesity and with increased serum PON1 activities in prepubertal children. We determined that lactonase is a reliable indicator of PON1 activities and should be included in future studies of PON1 function.

Key words: Paraoxonase 1 gene: Genetic polymorphisms: Obesity: Children

In humans, paraoxonase 1 (PON1) is primarily expressed in the liver and can be detected circulating in the plasma in association with HDL[(1)]. PON1 is involved in the metabolism of lipoprotein phospholipids and inhibits the lipid peroxidation of LDL and HDL[(1,2)]. Changes in PON1 activities have been shown to lead to a variety of diseases that involve oxidative stress, including CVD, Alzheimer's disease, chronic renal failure, the metabolic syndrome and chronic liver impairment[(3)].

With the prevalence of obesity and metabolic disorders, such as type 2 diabetes, being on the rise, these conditions will probably remain the major contributors to cardiovascular mortality and morbidity in the twenty-first century. A growing body of evidence that suggests that obesity-induced oxidative stress plays an important role in adults and children has emerged in the past few years. Recently, our group has demonstrated elevated levels of plasma myeloperoxidase (MPO) in prepubertal obese children, and we detected that the expression of this enzyme was correlated with biomarkers for inflammation and cardiovascular risk, such as high-sensitivity C-reactive protein, matrix metalloproteinase-9 and resistin[(4)]. Taken together, these findings highlight the need to investigate the molecular mechanisms linking metabolic stress, obesity and, ultimately, CVD. Furthermore, the finding that PON1 is expressed in the interstitial space of adipose tissue[(5)] suggests a role for the PON1 enzyme in these disorders.

Relatively little work has been conducted to address the role of PON1 in obesity, and the small number of studies that have been carried out are not always in agreement. For example,

Abbreviations: HDL-C, HDL-cholesterol; LD, linkage disequilibrium; MPO, myeloperoxidase; ox-LDL, oxidised LDL; PON1, paraoxonase 1.

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decreased levels of paraoxonase and arylesterase activities have been observed in obese adults[6,7] and children[8], although another study has observed no changes in paraoxonase activity in adults with the metabolic syndrome or obesity[9]. However, the lack of a strong link between PON1 activities and oxidative stress could be due to differences in measurement techniques and data analysis between these studies. For example, the actual physiological substrates of PON1 are unclear, and the available methods for testing PON1 activities use a variety of synthetic substrates, including paraoxon (paraoxonase activity), phenylacetate (arylesterase activity) and diazoxon (diazoxonase activity). Although less commonly reported in the literature, researchers have also tested PON1 lactonase activity using a variety of lactones, such as dihydrocoumarin. This activity has been proposed to be responsible for oxidised-lipid hydrolysis, HDL-mediated macrophage cholesterol efflux[10] and homocysteine–thiolactone hydrolysis[11]. Due to these technical issues, measurements of PON1 activities will probably differ depending on the specific substrate used[12,13]. Another issue is that serum PON1 activities are strongly affected by enzymatic genotype as well as by exogenous factors, such as age, diet and drug, alcohol and tobacco use[14]. Of these factors, genetics are the main determinant of PON1 variability, and PON1 activities can differ by as much as a factor of 40 across a population of healthy individuals from the same ethnic group[15]. Although nearly 200 SNP have been described for *PON1*[16], it is common for authors to state only two known coding polymorphisms in this gene: Q192R (rs662) and L55M (rs854560). To date, no strong links have been observed between the presence of these SNP and obesity, and although a number of association studies have been performed[17–19], the findings of these studies are inconsistent. With this in mind, we carried out a comprehensive association study in obese prepubertal children that had additional *PON1* SNP as well as the previously characterised missense variations. In addition, we measured serum PON1 activities using four different substrates (dihydrocoumarin, paraoxon, phenylacetate and diazoxon) and correlated these results with a wide variety of plasma biomarkers, including markers for oxidative stress, insulin resistance, obesity and cardiovascular risk, to determine their association with PON1 activities and genetic variability.

Experimental methods

Study population

In the present case–control study, we recruited 179 obese children (95 males and 84 females) and 189 normal-weight children (109 males and 80 females), aged 4–13 years, from two Spanish cities (Córdoba and Santiago de Compostela). Childhood obesity was defined according to Cole *et al.*[20]. To be included in the study, it was required that the children were prepubertal and were not suffering from nutritional diseases or endogenous obesity. Children who were suffering from disease or malnutrition, currently in puberty or using medications to alter blood pressure, glucose levels or lipid metabolism were excluded from the study. Following an initial assessment at school or a primary-care centre, children who met the inclusion criteria were invited for a clinical examination at a participating hospital. The parents or guardians of the children were informed concerning the purpose and procedures of the study before written consent was obtained; all of the children agreed to participate in the study. Sex hormone levels were measured to confirm that the children were prepubertal (data not shown). The protocol was performed in accordance with the Declaration of Helsinki (Edinburgh 2000, revised), the recommendations of the Good Clinical Practice of the CEE (Document 111/3976/88, July, 1990) and the current Spanish regulations dictating clinical investigations in human subjects (RD 223/04 on Clinical Assays). The present study was approved by the Ethics Committee on Human Research of the University of Granada, the Ethics Committee of the Reina Sofía University Hospital of Córdoba and the Bioethics Committee of the University of Santiago de Compostela.

Anthropometric and biochemical measurements

Anthropometric measurements were performed by a single examiner with the children in bare feet and dressed in their underwear. Body weight (kg), height (cm) and waist circumference (cm) were measured using standardised procedures and were used to calculate the BMI of the children. Obesity was defined according to BMI, using the age- and sex-specific cut-off points proposed by Cole *et al.*[20] (linked to adult cut-offs of 25 and 30 kg/m^2). Blood pressure was measured three times for each individual by the same examiner, according to international recommendations. Blood samples were drawn from the antecubital vein after the children had fasted overnight. Biochemical analyses and measurements of specific biomarkers, including adiponectin, leptin, oxidised LDL (ox-LDL) and high-sensitivity C-reactive protein, were performed as described previously[21]. The levels of MPO, matrix metalloproteinase-9 and both the total and active forms of plasminogen activator inhibitor-1 were measured as described previously[4]. Plasma total antioxidant capacity was assessed using the spectrophotometric antioxidant assay kit from Cayman (catalogue no. 709001). Fatty acid-binding protein 4 concentrations were measured using ELISA (catalogue no. RD191036200R; BioVendor). Quantitative insulin sensitivity check index and homeostasis model assessment for insulin resistance scores were calculated using plasma glucose and insulin levels. Retinol, α-tocopherol and β-carotene levels were analysed using HPLC, as described previously[22].

DNA isolation and genotyping

Genomic DNA was extracted using the QIAamp Blood kit (Qiagen). Based on their location with respect to the *PON1* gene, a total of sixteen SNP were selected from the HapMap and NCBI databases. First, each missense variation was selected. Then, SNP were selected from the promoter 3′ untranslated region (UTR) and 5′UTR. All SNP had minor allele frequencies greater than 0·05 in the Caucasian

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population. Table S1 (available online) describes the main characteristics of the SNP included in the present study.

Genotyping was performed using the Illumina GoldenGate Assay (Illumina), as described previously[21]. The success rates for genotyping were >95 % for nearly all of the SNP, with the exception of rs705379, which was excluded from further analysis. The Hardy–Weinberg equilibrium for each SNP was determined with the exact test using the PLINK software program (version 1.07; http://pngu.mgh.harvard.edu/~purcell/plink)[23]. The Hardy–Weinberg equilibrium *P* values were greater than 0·05 in both the obese and normal-weight groups for all the SNP, and the observed allele frequencies of the SNP in the present study were similar to those reported in the HapMap database for Caucasians (Table S1, available online). Linkage disequilibrium (LD) was analysed using the Haploview 4.2 software program (http://www.broad.mit.edu/mpg/haploview/)[24].

Determination of paraoxonase 1 activities

Serum PON1 activities were determined using four different substrates (dihydrocoumarin, paraoxon, phenylacetate and diazoxon), as described previously[15,25]. Arylesterase, diazoxonase and lactonase enzyme assays were performed using a Lambda-2 spectrophotometer (Perkin Elmer Inc.). PON1 paraoxonase activity was determined using a multidetection microplate reader (BioTek Synergy HT; BioTek Instruments Inc.). The samples were assayed in duplicate, and the average value was used for further analysis. The non-enzymatic hydrolysis rate for each substrate was measured by substituting serum for buffer, and this value was subtracted from the total rate of hydrolysis.

Statistical analyses

All of the statistical analyses were performed using either the PLINK or SPSS software program (version 15.0.1; SPSS, Inc.). The normal distribution of the clinical parameter data was assessed using the Kolmogorov–Smirnov test. Normally distributed continuous variables are expressed as means with their standard errors, and non-normally distributed variables are expressed as medians and ranges. The values of glucose, TAG, leptin, α-tocopherol, β-carotene and ox-LDL were logarithmically transformed to approximate normal distributions; the values of insulin, homeostasis model assessment for insulin resistance and adiponectin were transformed using the square-root transformation technique, and the quantitative insulin sensitivity check index was inversely transformed. Mean comparisons between continuous variables for the obese and normal-weight children were performed using Student's *t* test for unpaired samples. The paraoxonase activity could not be transformed to a normal distribution due to its bimodal nature; therefore, the Mann–Whitney *U* test was applied.

Genotypic relative risk was assessed by comparing the obese group with the normal-weight group and by calculating the OR and the 95 % CI using a logistic regression analysis under an additive model adjusted for age and sex. A meta-analysis was performed to discard population stratification due to the two cities the children were recruited from.

Results

Patient characteristics

Table 1 summarises the demographic, clinical and biochemical characteristics of the study population. As expected, the obese children had significantly greater values for weight, BMI, BMI *z*-score, waist circumference, leptin and fatty acid-binding protein 4 and significantly lower adiponectin levels. Metabolic syndrome parameters, such as systolic and diastolic blood pressure, TAG, insulin and homeostasis model assessment for insulin resistance values, were significantly higher in the obese children, whereas the quantitative insulin sensitivity check index, HDL-cholesterol (HDL-C) and ApoA1 values were lower in this group. The levels of the cardiovascular risk biomarkers MPO, active plasminogen activator inhibitor-1 and total plasminogen activator inhibitor-1 were significantly increased in the obese children, whereas no difference was observed for that of matrix metalloproteinase-9 compared with that in the normal-weight group. The levels of the oxidative stress biomarkers ox-LDL, retinol and α-tocopherol were not significantly different between the groups, whereas those of β-carotene and total antioxidant capacity were lower in the obese children. Inflammation was also detected in the obese group, as indicated by greater high-sensitivity C-reactive protein values.

Paraoxonase 1 activities in childhood obesity and their correlations with metabolic, cardiovascular risk and oxidative stress biomarkers

None of the PON1 activities differed between the obese and normal-weight children (Table S2, available online).

Lactonase activity showed significant correlations with the levels of the greatest number of analysed biomarkers compared with the other PON1 activities (Table 2). Lactonase activity was positively correlated with the levels of several biomarkers involved in lipid metabolism, including total cholesterol, LDL-cholesterol, HDL-C and ApoA1. Moreover, lactonase activity was positively correlated with the levels of several antioxidant molecules, including retinol and α-tocopherol, and it was negatively correlated with those of fatty acid-binding protein 4 and MPO, which have recently been described as biomarkers for CVD risk.

With respect to the activities of the other enzymes, total cholesterol, HDL-C and ApoA1 levels were correlated with diazoxonase (r 0·174, $P<0·001$; r 0·247, $P<0·001$; r 0·121, $P=0·022$, respectively) and arylesterase (r 0·232, $P<0·001$; r 0·222, $P<0·001$; r 0·225, $P<0·001$, respectively) activities. In addition, retinol levels were positively correlated with lactonase and arylesterase (r 0·130, $P=0·014$) activities. However, paraoxonase activity was not significantly correlated with any of the tested biomarker levels or anthropometric measurements; similar results were observed in both the experimental and control groups (data not shown). No significant correlations were observed between any of the PON1 activities and weight, BMI, BMI *z*-score, waist circumference, DBP, glucose, insulin, quantitative insulin sensitivity check index, homeostasis model assessment for insulin resistance, TAG,

Table 1. Demographic, clinical and biochemical characteristics of the children included in the present study (Mean values with their standard errors)

	Normal weight		Obese		
Variables	Mean	SEM	Mean	SEM	*P**
Sex (*n*)					
Male	109		95		
Female	80		84		
Age (years)	8·9	0·1	8·6	0·1	0·106
Weight (kg)	29·1	0·4	51·0	1·0	<0·001
BMI (kg/m^2)	16·61	0·11	26·85	0·28	<0·001
BMI *z*-score	−0·19	0·04	3·52	0·11	<0·001
WC (cm)	58·2	0·5	81·0	1·0	<0·001
SBP (mmHg)	96	1	110	1	<0·001
DBP (mmHg)	58	1	69	1	<0·001
Glucose (mg/l)	830	10	840	10	0·778
Insulin (pmol/l)	35·88	1·38	71·76	3·45	<0·001
QUICKI	0·390	0·003	0·351	0·002	<0·001
HOMA-IR	1·08	0·04	2·17	0·11	<0·001
Total cholesterol (mg/l)	1720	20	1660	20	0·052
TAG (mg/l)	540	10	760	30	<0·001
HDL-C (mg/l)	650	10	520	10	<0·001
LDL-C (mg/l)	940	20	970	20	0·227
ApoA1 (mg/l)	1510	20	1330	20	<0·001
ApoB (mg/l)	670	10	720	10	0·005
FABP-4 (ng/ml)	9·54	0·60	27·23	1·47	<0·001
Adiponectin (mg/l)	27·81	0·81	22·57	0·84	<0·001
Leptin (μg/l)	4·15	0·29	22·70	1·03	<0·001
ox-LDL (mg/l)	2·057	0·213	2·045	0·191	0·502
Retinol (μg/ml)	0·247	0·005	0·254	0·005	0·351
α-Tocopherol (μg/ml)	7·965	0·129	7·907	0·160	0·776
β-Carotene (μg/ml)	0·148	0·009	0·086	0·004	<0·001
TAC (mM-Trolox)	2·18	0·05	1·79	0·07	<0·001
hsCRP (mg/l)	0·986	0·285	3·520	0·286	<0·001
MMP-9 (μg/l)	83·21	3·72	94·40	5·58	0·815
MPO (μg/l)	13·84	1·43	24·63	2·60	<0·001
Active PAI-1 (μg/l)	4·73	0·26	12·70	0·78	<0·001
Total PAI-1 (μg/l)	18·54	0·95	29·84	1·56	<0·001

WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; QUICKI, quantitative insulin sensitivity check index; HOMA-IR, homeostasis model assessment for insulin resistance; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; FABP-4, fatty acid-binding protein 4; ox-LDL, oxidised LDL; TAC, total antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; hsCRP, high-sensitivity C-reactive protein; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; PAI-1, plasminogen activator inhibitor-1.

* Student's *t* test.

α-tocopherol, β-carotene, total antioxidant capacity, ox-LDL, high-sensitivity C-reactive protein, adiponectin or leptin values (data not shown).

We also examined the relationship between the different enzymatic activities within our cohort. Lactonase activity was closely associated with both arylesterase (r 0·895, $P<0·001$) and diazoxonase (r 0·673, $P<0·001$) activities. Arylesterase activity was also associated with diazoxonase activity (r 0·787, $P<0·001$). However, paraoxonase activity was only weakly correlated with lactonase activity (r 0·172, $P=0·001$).

Association of paraoxonase 1 SNP with the incidence of childhood obesity and related traits

The results of the logistic regression analysis and the SNP allele frequencies are given in Table 3. The only SNP that showed an association with obesity was rs854566, which showed a negative association (OR 0·61, 95% CI 0·41, 0·91; $P=0·016$) under the additive model of inheritance. This association was not significant after Bonferroni correction for multiple testing (data not shown). Other models of inheritance such as recessive model did not add strength to this association. Population stratification was discarded by conducting a meta-analysis using the individual association results from each of the examined populations (Córdoba and Santiago de Compostela) ($Q = 0·906$, $I = 0·00$). The missense SNP Q192R and L55M were distributed equally among the study group populations, and they did not show any association with obesity.

Phenotypic associations between the Q192R, L55M and rs854566 SNP and the anthropometric measurements as well as the biomarkers for metabolic, oxidative stress and cardiovascular risk are given in Table S3 (available online). The associations between SNP rs854566 and BMI, BMI *z*-score and weight were significant ($P<0·05$), although these associations lost their significance when the samples were divided into obese and normal-weight groups (data not shown). None of the oxidative stress biomarkers (ox-LDL, total antioxidant capacity, retinol, α-tocopherol and β-carotene) was

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Table 2. Correlations between serum paraoxonase 1 lactonase activity and metabolic, cardiovascular risk and oxidative stress biomarkers

Biomarkers	*r*	*P*
Weight (kg)	−0·057	0·274
BMI (kg/m^2)	−0·062	0·240
BMI *z*-score	−0·073	0·162
WC (cm)	0·005	0·923
SBP (mmHg)	−0·075	0·167
DBP (mmHg)	−0·077	0·154
Glucose (mg/l)	0·034	0·525
Insulin (pmol/l)	−0·075	0·160
QUICKI	−0·056	0·300
HOMA-IR	−0·073	0·174
Total cholesterol (mg/l)	0·248	<0·001
TAG (mg/l)	−0·037	0·480
HDL-C (mg/l)	0·235	<0·001
LDL-C (mg/l)	0·131	0·013
ApoA1 (mg/l)	0·254	<0·001
ApoB (mg/l)	0·078	0·138
FABP-4	−0·227	0·033
Adiponectin (mg/l)	0·079	0·137
Leptin (μg/l)	−0·024	0·650
ox-LDL (mg/l)	−0·043	0·410
Retinol (μg/ml)	0·259	<0·001
α-Tocopherol (μg/ml)	0·167	0·001
β-Carotene (μg/ml)	0·075	0·158
TAC (mM-Trolox)	−0·042	0·426
hsCRP (mg/l)	−0·006	0·915
MMP-9 (μg/l)	−0·063	0·230
MPO (μg/l)	−0·110	0·037
Active PAI-1 (μg/l)	0·012	0·826
Total PAI-1 (μg/l)	0·005	0·923

WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; QUICKI, quantitative insulin sensitivity check index; HOMA-IR, homeostasis model assessment for insulin resistance; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; FABP-4, fatty acid-binding protein 4; ox-LDL, oxidised LDL; TAC, total antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; hsCRP, high-sensitivity C-reactive protein; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; PAI-1, plasminogen activator inhibitor-1.

significantly associated with the examined SNP, which was also true for the insulin resistance and cardiovascular risk biomarkers.

Effects of paraoxonase 1 SNP on paraoxonase 1 activities

All of the examined SNP showed a certain effect on PON1 activities, with the exception of rs3735590, which is located in the 3′UTR (Table 4). The remaining SNP showed different association patterns that varied according to their position within the *PON1* gene and according to their LD values. The LD analysis (Fig. 1) identified three blocks defined by high r^2 values between the SNP.

SNP located in the promoter region (rs854571, rs854572, rs854573, rs705382, rs757158 and rs13236941) were strongly associated with lactonase, arylesterase and diazoxonase activities, whereas their associations with paraoxonase activity were much weaker; only SNP rs854572 and rs757158 showed significant associations with paraoxonase activity. Block 3 corresponds to the SNP located in the promoter region.

The intronic SNP rs854566, which was negatively associated with obesity, showed significant effects on all of the tested PON1 activities. The presence of the protective allele (A) was highly associated with increased diazoxonase, lactonase and arylesterase activities and more weakly associated with decreased paraoxonase activity. This SNP was not included in any block due to its weak LD values with neighbouring SNP.

With respect to the missense mutations, rs662 (Q192R) showed the greatest effect on paraoxonase activity, which was highly significant ($\beta = 131{\cdot}5$, 95% CI 115·0, 147·9; $P = 5{\cdot}88 \times 10^{-42}$). This variant was also excluded from the LD blocks due to its weak LD values ($r^2 < 0{\cdot}28$) with the other SNP. With respect to rs854560 (L55M), individuals with the 55LL genotype showed greater diazoxonase, lactonase, arylesterase and paraoxonase activities. The intronic SNP rs705378 shared the same associations as rs854560 due to

Table 3. Logistic regression analysis of the *PON1* SNP and obesity under the additive model of inheritance (Odds ratios and 95 % confidence intervals)

		Obese			Normal weight			MAF				
SNP	Allele 1/allele 2	11	12	22	11	12	22	Obese	Normal weight	OR*	95 % CI	*P* (additive model)†
rs13236941	G/A	135	40	2	137	38	5	0·124	0·133	0·95	0·62, 1·48	0·829
rs757158	G/A	75	79	23	72	80	29	0·353	0·381	0·89	0·66, 1·20	0·451
rs705382	G/C	86	78	13	88	70	23	0·294	0·320	0·90	0·67, 1·24	0·517
rs854573	A/G	109	63	5	108	63	10	0·206	0·229	0·88	0·61, 1·27	0·498
rs854572	G/C	70	81	26	68	78	35	0·376	0·409	0·88	0·66, 1·18	0·382
rs854571	G/A	97	74	6	97	71	13	0·243	0·268	0·87	0·62, 1·24	0·451
rs854566	G/A	126	50	1	112	59	10	0·147	0·218	0·62	0·41, 0·91	0·016
rs705378	C/A	60	90	27	68	83	30	0·407	0·395	1·04	0·77, 1·41	0·796
rs854560	A(Leu)/T(Met)	60	90	27	68	83	30	0·407	0·395	1·04	0·77, 1·41	0·796
rs3917527	A/G	165	12	0	161	20	0	0·034	0·055	0·57	0·27, 1·21	0·143
rs662	A(Gln)/G(Arg)	90	72	15	85	79	16	0·288	0·308	0·90	0·65, 1·24	0·518
rs854552	A/G	117	53	7	112	57	12	0·189	0·224	0·80	0·56, 1·14	0·217
rs854551	G/A	132	41	4	132	42	7	0·138	0·155	0·87	0·58, 1·30	0·488
rs3735590	G/A	161	16	0	156	25	0	0·045	0·069	0·60	0·31, 1·18	0·138
rs854550	G/A	133	40	44	133	41	7	0·136	0·152	0·86	0·57, 1·29	0·472

MAF, minor allele frequency; allele 1/2, major/minor allele.
* Adjusted for sex and age.
† From the logistic regression analysis.

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Table 4. Associations between the paraoxonase 1 (*PON1*) SNP and PON1 activities
(*β* Values and 95 % confidence intervals)

	Lactonase			Arylesterase			Diazoxonase			Paraoxonase		
SNP	*β**	95 % CI	*P*	*β**	95 % CI	*P*	*β**	95 % CI	*P*	*β**	95 % CI	*P*
rs13236941	0·96	0·51, 1·41	$3·86\times10^{-5}$	11·58	5·55, 17·60	$1·97\times10^{-4}$	0·64	0·22, 1·06	$3·28\times10^{-3}$	1·54	−27·57, 30·64	0·917
rs757158	1·16	0·87, 1·46	$7·78\times10^{-14}$	14·67	10·68, 18·65	$3·76\times10^{-12}$	0·94	0·66, 1·22	$1·50\times10^{-10}$	21·68	1·82, 41·54	0·033
rs705382	1·29	0·98, 1·59	$2·11\times10^{-15}$	17·01	12·94, 21·09	$5·84\times10^{-15}$	1·10	0·81, 1·39	$5·60\times10^{-13}$	−1·90	−22·87, 19·07	0·859
rs854573	1·23	0·87, 1·59	$9·07\times10^{-11}$	15·86	11·02, 20·69	$4·55\times10^{-10}$	1·09	0·75, 1·43	$8·18\times10^{-10}$	−4·42	−28·48, 19·64	0·719
rs854572	1·22	0·94, 1·50	$5·93\times10^{-16}$	15·50	11·68, 19·32	$2·78\times10^{-14}$	1·03	0·77, 1·30	$3·52\times10^{-13}$	21·51	2·20, 40·83	0·030
rs854571	1·22	0·87, 1·57	$2·06\times10^{-11}$	14·94	10·28, 19·60	$1·02\times10^{-9}$	0·98	0·65, 1·31	$1·09\times10^{-8}$	2·99	−20·14, 26·12	0·800
rs854566	1·03	0·63, 1·42	$4·68\times10^{-7}$	14·44	9·21, 19·66	$1·15\times10^{-7}$	1·20	0·84, 1·56	$1·93\times10^{-10}$	−37·77	−63·05, −12·50	$3·68\times10^{-3}$
rs705378	−0·88	−1·19, −0·57	$3·44\times10^{-8}$	−9·48	−13·66, −5·29	$1·25\times10^{-5}$	−0·91	−1·19, −0·62	$1·22\times10^{-9}$	−74·07	−92·68, −55·46	$7·57\times10^{-14}$
rs854560	−0·88	−1·19, −0·57	$3·44\times10^{-8}$	−9·48	−13·66, −5·29	$1·25\times10^{-5}$	−0·91	−1·19, −0·62	$1·22\times10^{-9}$	−74·07	−92·68, −55·46	$7·57\times10^{-14}$
rs3917527	−0·16	−0·92, 0·61	0·691	−4·98	−15·29, 5·32	0·344	−0·28	−1·00, 0·43	0·436	67·54	20·17, 114·90	$5·49\times10^{-3}$
rs662	−0·20	−0·55, 0·14	0·250	−7·94	−12·52, −3·37	$7·50\times10^{-4}$	−0·35	−0·67, −0·03	0·031	131·50	115·00, 147·90	$5·88\times10^{-42}$
rs854552	0·60	0·23, 0·97	$1·51\times10^{-3}$	6·92	1·98, 11·85	$6·35\times10^{-3}$	0·52	0·18, 0·86	$3·24\times10^{-3}$	55·66	33·02, 78·30	$2·18\times10^{-6}$
rs854551	0·82	0·40, 1·24	$1·40\times10^{-4}$	9·96	4·33, 15·59	$5·90\times10^{-4}$	0·77	0·38, 1·16	$1·17\times10^{-4}$	58·98	33·03, 84·93	$1·14\times10^{-5}$
rs3735590	−0·09	−0·78, 0·59	0·787	−2·36	−11·47, 6·45	0·612	−0·18	−0·82, 0·46	0·583	29·93	−12·87, 72·74	0·174
rs854550	0·80	0·38, 1·22	$2·26\times10^{-4}$	9·75	4·09, 15·41	$8·23\times10^{-3}$	0·77	0·38, 1·16	$1·37\times10^{-4}$	57·48	31·36, 83·60	$2·11\times10^{-5}$

*Variable change per minor allele, under the additive model adjusted for sex and age.

their high LD (r^2 1) value. These two SNP correspond to block 2.

SNP located in the *PON1* 3'UTR (rs854550, rs854551 and rs854552) comprise block 1, and they were highly associated with paraoxonase activity and weakly associated with lactonase, arylesterase and diazoxonase activities. The intronic SNP rs3917527 was only associated with paraoxonase activity, and it was also excluded from the LD blocks.

Discussion

Many previous studies have investigated the association between *PON1* genetic variations and the risk for certain diseases or changes in PON1 activities in the context of certain diseases; however, no study has considered both variables simultaneously. To date, this is the first study to demonstrate genetic associations between *PON1* polymorphisms and prepubertal childhood obesity. Furthermore, the present study has used both intronic and exonic polymorphisms and four different substrates to measure serum PON1 activities. An association between obesity and the *PON1* SNP rs854566 has not been reported previously, and we found that it was negatively associated with childhood obesity and also affected all four of the measurements of PON1 activities. Children carrying the minor allele of SNP rs854566 had higher diazoxonase, lactonase and arylesterase activities and lower paraoxonase activity. In contrast, the genetic variants Q192R and L55M were not significantly associated with childhood obesity, although they did exert effects on PON1 activities. To the best of our knowledge, three previous studies have tested for genetic associations between the *PON1* gene and obesity. One study conducted on adolescents has reported no association between obesity and the Q192R polymorphism[18]. Another study conducted on obese Portuguese women has shown a positive association between the Q192R genotype and disease risk[17]. Lastly, a recent study conducted on obese Mexican adults has identified a positive association between the 55LL genotype and obesity[19]. However, these findings are inconsistent with those of the present study. It is likely that the complex nature of obesity, which depends on both genetic and environmental factors, is the cause for the unclear relationship between *PON1* genetic variations, PON1 activities and childhood obesity. The importance of conducting the present study in prepubertal children should be noted as this population should have lower exposure levels to toxins (e.g. tobacco, alcohol and drugs) than adults and is much less likely to have chronic diseases that might affect PON1 activities.

PON1 activities were not found to be significantly altered in the obese group based on any of the metrics that we used. One study has reported lowered paraoxonase activity in obese adults[6], whereas no changes in paraoxonase and arylesterase activities have been observed in another study[9]. The previous study that was conducted on children reported only measured paraoxonase and arylesterase activities, which were observed to be lower in fifty-nine obese children (11·95 (SEM 1·61) years) than in fifty-one normal-weight children (12·00 (SEM 3·91) years)[8]. However, the fact that this

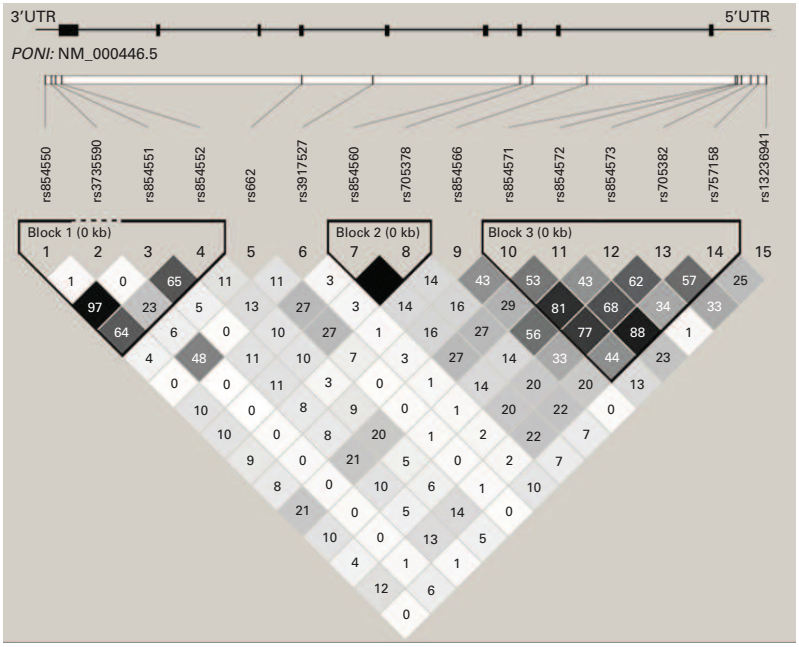

Fig. 1. Linkage disequilibrium diagram of the studied variants with the exon–intron scheme of the *PON1* gene. The r^2 values between the genotyped SNP are shown in each cell; black cells correspond to r^2 1·00. Block definition followed the four-gamete rule using the Haploview software.

population was not defined as entirely prepubertal, as well as the relatively low sample size, could explain the divergent results. Furthermore, the fact that lactonase activity had never been measured in obese children is significant and should be addressed.

Interestingly, we determined a negative correlation between lactonase activity and the level of MPO, which is considered to be a reliable biomarker for endothelial dysfunction and CVD risk[4]. Moreover, we observed a negative correlation between lactonase activity and the level of FAPB-4, a biomarker that has been linked to insulin resistance, the metabolic syndrome, hypertriacylglycerolaemia and atherosclerosis[26]. Therefore, the lack of any relationship between paraoxonase activity and the levels of these biomarkers supports the hypothesis that measuring other PON1 activities, rather than the paraoxonase activity, is more physiologically relevant for determining PON1 function[12]. Similarly, lactonase, arylesterase and diazoxonase activities were correlated with HDL-C, total cholesterol and ApoA1 levels, whereas paraoxonase activity was not. In this line, a recent study[27] has shown that arylesterase activity is a more powerful indicator of cardiovascular risk than paraoxonase activity. Considering the present results and the previously published evidence, we propose that lactonase activity is a good indicator of PON1 function and that it should be used in further studies investigating the role of PON1 in the development of obesity and CVD.

Although the protective effects of SNP rs854566 on childhood obesity need to be validated, it should be noted that this allele was associated with increased lactonase activity, which itself was positively correlated with HDL-C, ApoA1, retinol and α-tocopherol levels and negatively correlated with fatty acid-binding protein 4 and MPO levels. These relationships support a protective role for SNP rs854566 by linking the antioxidant and anti-inflammatory functions of PON1 with protection from the development of obesity and CVD. Indeed, it is known that HDL has anti-inflammatory and antioxidant functions *in vivo*, and it is believed from previous studies carried out in animal models that PON1 contributes to these functions[2,28]. However, although lactonase activity is most probably responsible for the antioxidant effects of PON1[10,11,29], we did not observe significant correlations with ox-LDL levels. The present findings are consistent with those of Carlson *et al.*[30], who reported no association between ox-LDL levels and arylesterase activity.

PON1 is expressed in the interstitial space of adipose tissue[3]. Mature adipocytes have been reported to express cluster of differentiation 36 (CD36), which can recognise and bind to ox-LDL; these molecules are then endocytosed[31] and can modulate adipose tissue mass by stimulating adipocyte proliferation and differentiation[32]. However, the function of PON1 in adipose tissue is unknown. Although we did not identify any relationship between PON1 activities and ox-LDL levels in the plasma, more studies should be performed to describe the effect of PON1 on ox-LDL levels in adipose tissue and to determine whether PON1 plays a role in adipocyte differentiation and proliferation during the development of obesity.

Several limitations in the present study should be noted. The negative association between the *PON1* SNP rs854566

and childhood obesity identified in the present study requires further validation in a larger population. Another potential limitation is that the measurements were only carried out under fasting conditions at a single time point. We, therefore, were unable to determine the variability and prognostic value of changing levels over time and of the impact of dietary or therapeutic interventions on serum PON1 activities.

Conclusion

The present results suggest a protective role for SNP rs854566 with respect to obesity. This SNP also exhibits strong associations with serum PON1 activities, namely increased diazoxonase, lactonase and arylesterase activities. Despite these results, serum PON1 activities were not found to be significantly different in prepubertal obese children. Nevertheless, we demonstrate that lactonase activity is a reliable indicator of PON1 function and should be used in future studies investigating the role of PON1 in the development of obesity and CVD. Future *in vivo* and *in vitro* studies on the function of PON1 in adipose tissue may help us to identify potential antioxidant and protective roles for PON1 in the protection against the development of obesity and its co-morbidities.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513001967

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The authors declare no conflicts of interest that could be perceived as affecting the impartiality of this research.

References

1. Mackness MI, Arrol S, Abbot C, *et al.* (1993) Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* **104**, 129–135.
2. Mackness MI, Durrington PN & Mackness B (2000) How high-density lipoprotein protects against the effects of lipid peroxidation. *Curr Opin Lipidol* **11**, 383–388.
3. Marsillach J, Parra S, Ferré N, *et al.* (2008) Paraoxonase-1 in chronic liver diseases, neurological diseases, and HIV infection. In *The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism*, pp. 187–198 [B Mackness, M Mackness, M Aviram and G Paragh, editors]. Dordrecht: Springer.
4. Olza J, Aguilera CM, Gil-Campos M, *et al.* (2012) Myeloperoxidase is an early biomarker of inflammation and cardiovascular risk in prepubertal obese children. *Diabetes Care* **35**, 2373–2376.
5. Marsillach J, Mackness B, Mackness M, *et al.* (2008) Immunohistochemical analysis of paraoxonases-1,2 and 3 expression in normal mouse tissues. *Free Radic Biol Med* **45**, 146–157.
6. Aslan M, Horoz M, Sabuncu T, *et al.* (2011) Serum paraoxonase enzyme activity and oxidative stress in obese subjects. *Pol Arch Med Wewn* **121**, 181–186.
7. Ferretti G, Bacchetti T, Moroni C, *et al.* (2005) Paraoxonase activity in high-density lipoproteins: a comparison between healthy and obese females. *J Clin Endocrinol Metab* **90**, 1728–1733.
8. Koncsos P, Seres I, Harangi M, *et al.* (2010) Human paraoxonase-1 activity in childhood obesity and its relation to leptin and adiponectin levels. *Pediatr Res* **67**, 309–313.
9. Tabur S, Torun AN, Sabuncu T, *et al.* (2010) Non-diabetic metabolic syndrome and obesity do not affect serum paraoxonase and arylesterase activities but do affect oxidative stress and inflammation. *Eur J Endocrinol* **162**, 535–541.
10. Rosenblat M, Gaidukov L, Khersonsky O, *et al.* (2006) The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. *J Biol Chem* **281**, 7657–7665.
11. Jakubowski H (2000) Calcium-dependent human serum homocysteine thiolactone hydrolase. A protective mechanism against protein N-homocysteinylation. *J Biol Chem* **275**, 3957–3962.
12. Camps J, Marsillach J & Joven J (2009) The paraoxonases: role in human diseases and methodological difficulties in measurement. *Crit Rev Clin Lab Sci* **46**, 83–106.
13. Camps J, Mackness M, Mackness B, *et al.* (2010) Serum paraoxonase-1 activity and genetic polymorphisms: common errors in measurement and interpretation of results. *Clin Chem Lab Med* **48**, 893–894.
14. Costa LG, Giordano G & Furlong CE (2011) Research update: pharmacological and dietary modulators of paraoxonase 1 (PON1) activity and expression: the hunt goes on. *Biochem Pharmacol* **81**, 337–344.
15. Richter RJ & Furlong CE (1999) Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics* **9**, 745–753.
16. Furlong CE, Richter RJ, Li WF, *et al.* (2008) The functional consequences of polymorphisms in the human *PON1* gene. In *The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism*, pp. 267–281 [B Mackness, M Mackness, M Aviram and G Paragh, editors]. Dordrecht: Springer.
17. Veiga L, Silva-Nunes J, Melao A, *et al.* (2011) Q192R polymorphism of the paraoxonase-1 gene as a risk factor for obesity in Portuguese women. *Eur J Endocrinol* **164**, 213–218.
18. Baráth A, Németh I, Karg E, *et al.* (2006) Roles of paraoxonase and oxidative stress in adolescents with uraemic, essential or

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obesity-induced hypertension. *Kidney Blood Press Res* **29**, 144–151.
19. Martínez-Salazar MF, Almenares-López D, García-Jiménez S, *et al.* (2011) Relationship between the paraoxonase (PON1) L55M and Q192R polymorphisms and obesity in a Mexican population: a pilot study. *Genes Nutr* **6**, 361–368.
20. Cole TJ, Bellizzi MC, Flegal KM, *et al.* (2000) Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* **320**, 1240–1243.
21. Olza J, Gil-Campos M, Leis R, *et al.* (2012) A gene variant of 11β-hydroxysteroid dehydrogenase type 1 is associated with obesity in children. *Int J Obes* **36**, 1558–1563.
22. Garcia-Rodriguez CE, Mesa MD, Olza J, *et al.* (2012) Does consumption of two portions of salmon per week enhance the antioxidant defense system in pregnant women? *Antioxid Redox Signal* **16**, 1401–1406.
23. Purcell S, Neale B, Todd-Brown K, *et al.* (2007) PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am J Hum Genet* **81**, 559–575.
24. Barrett JC, Fry B, Maller J, *et al.* (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265.
25. Hernández AF, Gil F, Leno E, *et al.* (2009) Interaction between human serum esterases and environmental metal compounds. *Neurotoxicology* **30**, 628–635.
26. Kralisch S & Fasshauer M (2012) Adipocyte fatty acid binding protein: a novel adipokine involved in the pathogenesis of metabolic and vascular disease? *Diabetologia* **56**, 10–21.
27. Tang WH, Hartiala J, Fan Y, *et al.* (2012) Clinical and genetic association of serum paraoxonase and arylesterase activities with cardiovascular risk. *Arterioscler Thromb Vasc Biol* **32**, 2803–2812.
28. Mackness B, Quarck R, Verreth W, *et al.* (2006) Human paraoxonase-1 overexpression inhibits atherosclerosis in a mouse model of metabolic syndrome. *Arterioscler Thromb Vasc Biol* **26**, 1545–1550.
29. Gaidukov L & Tawfik DS (2007) The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity. *J Lipid Res* **48**, 1637–1646.
30. Carlson CS, Heagerty PJ, Hatsukami TS, *et al.* (2006) TagSNP analyses of the *PON* gene cluster: effects on PON1 activity, LDL oxidative susceptibility, and vascular disease. *J Lipid Res* **47**, 1014–1024.
31. Kuniyasu A, Hayashi S & Nakayama H (2002) Adipocytes recognize and degrade oxidized low density lipoprotein through CD36. *Biochem Biophys Res Commun* **295**, 319–323.
32. Masella R, Vari R, D'Archivio M, *et al.* (2006) Oxidised LDL modulate adipogenesis in 3T3-L1 preadipocytes by affecting the balance between cell proliferation and differentiation. *FEBS Lett* **580**, 2421–2429.

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Table S1. Characteristics of the genotyped SNPs.

SNP	BP	Major allele	Minor allele	Location	MAF Hapmap	MAF Normal-weight	HW *P*-value
rs13236941	94793756	G	A	5' UTR	0.153	0.124	0.324
rs757158	94793464	G	A	5' UTR	0.408	0.353	0.431
rs705382	94793157	G	C	5' UTR	0.297	0.294	0.128
rs854573	94792799	A	G	5' UTR	0.195	0.206	0.834
rs854572	94792632	G	C	5' UTR	0.441	0.376	0.166
rs854571	94792555	G	A	5' UTR	0.267	0.243	1.000
rs705379	94791831	A	G	5’ UTR	0.409	EA	EA
rs854566	94786685	G	A	Intron 1	0.117	0.147	0.518
rs705378	94784507	C	A	Intron 2	0.374	0.407	0.641
rs854560	94784020	A	T	Missense, Exon 3	0.397	0.407	0.641
rs3917527	94778194	A	G	Intron 5	0.050	0.034	1.000
rs662	94775382	A	G	Missense, Exon 6	0.358	0.288	0.861
rs854552	94765860	A	G	3' UTR	0.268	0.189	0.203
rs854551	94765613	G	A	3' UTR	0.203	0.138	0.150
rs3735590	94765431	G	A	3' UTR	0.051	0.045	1.000
rs854550	94765178	G	A	3' UTR	0.216	0.136	0.141

BP: base pair position; CHISQ: Chi-square statistic; EA: excluded from analysis; HW: Hardy Weinberg; MAF: minor allele frequency.

4 Table S2. Association of SNPs Q192R, L55M and rs854566 with anthropometric, insulin resistance, oxidative stress and obesity biomarkers.

	rs854566		**Q192R**		**L55M**	
	β(95% CI)	***P***	**β(95% CI)**	***P***	**β(95% CI)**	***P***
Weight (kg)	**-3.03(-5.58, -0.48)**	**0.020**	-0.41(-2.57, 1.75)	0.712	-0.27(-2.28, 1.73)	0.789
BMI	**-1.32(-2.43, -0.21)**	**0.020**	-0.25(-1.18, 0.69)	0.608	-0.18(-1.05, 0.69)	0.691
BMI Z-score	**-0.536(-0.934, -0.137)**	**0.009**	-0.154(-0.492, 0.184)	0.371	0.015(-0.300, 0.330)	0.924
WC (cm)	-4.36(-8.74, 0.02)	0.052	-1.34(-5.05, 2.36)	0.478	0.14(-3.30, 3.56)	0.936
SBP (mmHg)	-0.21(-3.15, 2.72)	0.886	0.92(-1.55, 3.42)	0.464	-1.34(-3.62, 0.95)	0.252
DBP (mmHg)	-1.55(-3.98, 0.88)	0.213	1.02(-1.05, 3.09)	0.335	-0.98(-2.88, 0.91)	0.312
Glucose (mg/dL)	0.28(-1.05, 1.56)	0.683	0.59(-0.52, -1.70)	0.299	-0.19(-1.23, 0.84)	0.712
Insulin (mU/L)	-0.89(-2.05, 0.27)	0.133	-0.18(-1.16, 0.79)	0.711	0.23(-0.68, 1.14)	0.619
QUICKI	0.007(-0.001, 0.014)	0.096	-0.002(-0.008, 0.004)	0.753	0.0003(-0.005, 0.006)	0.957
HOMA-IR	-0.14(-0.38, 0.09)	0.096	-0.02(-0.23, 0.18)	0.662	0.06(-0.13, 0.24)	0.959
Total cholesterol (mg/dL)	1.47(-4.22, 7.16)	0.751	-0.93(-5, 72, 3.86)	0.499	-0.81(-5.26, 3.64)	0.950
TAG (mg/dL)	-3.56(-9.72, 2.60)	0.258	-0.48(-5.67, 4.71)	0.856	-0.81(-5.64, 4.01)	0.741
HDL-c (mg/dL)	1.08(-1.79, 3.95)	0.461	0.21(-2.20, 2.63)	0.862	-0.72(-2.97, 1.52)	0.529
LDL-c (mg/dL)	0.20(-4.69, 5.09)	0.936	-0.49(-4.60, 3.61)	0.813	0.26(-3.56, 4.08)	0.893
ApoA1 (mg/dL)	-1.06(-6.28, 4.17)	0.692	0.74(-3.68, 5.16)	0.742	0.45(-3.64, 4.55)	0.828
ApoB (mg/dL)	-0.56(-4.06, 2.94)	0.754	0.53(-2.42, 3.48)	0.725	-0.45(-3.19, 2.28)	0.746
FABP-4 (ng/mL)	-1.70(-5.62, 2.22)	0.378	0.88(-2.74, 4.50)	0.635	-1.06(-4.21, 2.09)	0.511
Adiponectin (mg/L)	0.83(-1.38, 3.03)	0.464	0.83(-1.38, 3.03)	0.464	-0.39(-2.13, 1.34)	0.657
Leptin (μg/L)	-2.33(-4.96, 0.30)	0.083	-1.53(-3.75, 0.68)	0.176	0.89(-1.19, 2.97)	0.401

ox-LDL (mg/L)	0.161(-0.361, 0.684)	0.545	-0.157(-0.600, 0.286)	0.487	0.207(-0.202, 0.616)	0.322
Vitamin A (μg/mL)	-0.006(-0.02, 0.007)	0.382	-0.007(-0.017, 0.004)	0.212	0.004(-0.006, 0.014)	0.404
α-tocopherol(μg/mL)	-0.08(-0.46, 0.30)	0.669	0.06(-0.26, 0.38)	0.715	-0.06(-0.36, 0.23)	0.672
β-carotene (μg/mL)	0.01(-0.007, 0.03)	0.220	-0.01(-0.02, 0.01)	0.190	0.002(-0.01, 0.01)	0.833
TAC (mM Trolox)	-0.08(-0.24, 0.08)	0.349	0.08(-0.06, 0.21)	0.277	0.08(-0.05, 0.20)	0.248
hsCRP (mg/L)	-0.23(-1.01, 0.56)	0.571	-0.36(-1.02, 0.30)	0.289	0.20(-0.41, 0.80)	0.522
MMP-9 (μg/L)	4.55(-7.72, 16.83)	0.467	-1.88(-12.19, 8.42)	0.720	0.27(-9.33, 0.87)	0.957
MPO (μg/L)	-2.21(-7.06, 2.65)	0.374	1.55(-2.53, 5.63)	0.458	-0.79(-4.58, 3.01)	0.685
Active PAI-1 (μg/L)	-1.54(-3.20, 0.11)	0.069	0.81(-0.59, 2.20)	0.258	0.08(-1.22, 1.38)	0.905
Total PAI-1 (μg/L)	-2.44(-5.80, 0.92)	0.159	1.42(-1.39, 4.24)	0.223	-0.21(-2.84, 2.43)	0.814

DBP: diastolic blood pressure; FABP-4: fatty acid binding protein 4; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment for insulin resistance; hsCRP: high-sensitivity C-reactive protein; LDL-C: low-density lipoprotein cholesterol; ox-LDL: oxidized LDL; SBP: systolic blood pressure; TAC: total antioxidant capacity; QUICKI: quantitative insulin sensitivity check index; WC: waist circumference. Insulin, cholesterol and HOMA-IR were logarithmically transformed, and QUICKI was inversely transformed to reach a normal distribution.

β: variable change per minor allele, adjusted by sex and age under the additive model.

Table S3. Association of SNPs with PON1 activities.

SNP	Lactonase		Arylesterase		Diazoxonase		Paraoxonase	
	β(95% CI)	*P*	β(95% CI)	*P*	β(95% CI)	*P*	β(95% CI)	*P*
rs13236941	0.96(0.51, 1.41)	3.86×10^{-5}	11.58(5.55, 17.60)	1.97×10^{-4}	0.64(0.22, 1.06)	3.28×10^{-3}	1.54(-27.57, 30.64)	0.917
rs757158	1.16(0.87, 1.46)	7.78×10^{-14}	14.67(10.68, 18.65)	3.76×10^{-12}	0.94(0.66, 1.22)	1.50×10^{-10}	21.68(1.82, 41.54)	0.033
rs705382	1.29(0.98, 1.59)	2.11×10^{-15}	17.01(12.94, 21.09)	5.84×10^{-15}	1.10(0.81, 1.39)	5.60×10^{-13}	-1.90(-22.87, 19.07)	0.859
rs854573	1.23(0.87, 1.59)	9.07×10^{-11}	15.86(11.02, 20.69)	4.55×10^{-10}	1.09(0.75, 1.43)	8.18×10^{-10}	-4.42(-28.48, 19.64)	0.719
rs854572	1.22(0.94, 1.50)	5.93×10^{-16}	15.50(11.68, 19.32)	2.78×10^{-14}	1.03(0.77, 1.30)	3.52×10^{-13}	21.51(2.20, 40.83)	0.030
rs854571	1.22(0.87, 1.57)	2.06×10^{-11}	14.94(10.28, 19.60)	1.02×10^{-9}	0.98(0.65, 1.31)	1.09×10^{-8}	2.99(-20.14, 26.12)	0.800
rs854566	1.03(0.63, 1.42)	4.68×10^{-7}	14.44(9.21, 19.66)	1.15×10^{-7}	1.20(0.84, 1.56)	1.93×10^{-10}	-37.77(-63.05, -12.50)	3.68×10^{-3}
rs705378	-0.88(-1.19, -0.57)	3.44×10^{-8}	-9.48(-13.66, -5.29)	1.25×10^{-5}	-0.91(-1.19, -0.62)	1.22×10^{-9}	-74.07(-92.68, -55.46)	7.57×10^{-14}
rs854560	-0.88(-1.19, -0.57)	3.44×10^{-8}	-9.48(-13.66, -5.29)	1.25×10^{-5}	-0.91(-1.19, -0.62)	1.22×10^{-9}	-74.07(-92.68, -55.46)	7.57×10^{-14}
rs3917527	-0.16(-0.92, 0.61)	0.691	-4.98(-15.29, 5.32)	0.344	-0.28(-1.00, 0.43)	0.436	67.54(20.17, 114.90)	5.49×10^{-3}
rs662	-0.20(-0.55, 0.14)	0.250	-7.94(-12.52, -3.37)	7.50×10^{-4}	-0.35(-0.67, -0.03)	0.031	131.50(115.00, 147.90)	5.88×10^{-42}
rs854552	0.60(0.23, 0.97)	1.51×10^{-3}	6.92(1.98, 11.85)	6.35×10^{-3}	0.52(0.18, 0.86)	3.24×10^{-3}	55.66(33.02, 78.30)	2.18×10^{-6}
rs854551	0.82(0.40, 1.24)	1.40×10^{-4}	9.96(4.33, 15.59)	5.90×10^{-4}	0.77(0.38, 1.16)	1.17×10^{-4}	58.98(33.03, 84.93)	1.14×10^{-5}
rs3735590	-0.09(-0.78, 0.59)	0.787	-2.36(-11.47, 6.45)	0.612	-0.18(-0.82, 0.46)	0.583	29.93(-12.87, 72.74)	0.174
rs854550	0.80(0.38, 1.22)	2.26×10^{-4}	9.75(4.09, 15.41)	8.23×10^{-3}	0.77(0.38, 1.16)	1.37×10^{-4}	57.48(31.36, 83.60)	2.11×10^{-5}

β: variable change per minor allele, adjusted by sex and age under the additive model.

Chapter 4

Are Catalase -844A/G Polymorphism and Activity Associated with Childhood Obesity?

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NEWS & VIEWS

Are Catalase −844A/G Polymorphism and Activity Associated with Childhood Obesity?

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Abstract

Catalase (CAT) is a peroxisomal antioxidant enzyme that is up-regulated upon oxidative stress. Previous studies have found associations between some single nucleotide polymorphisms (SNPs) located in the *CAT* promoter region in a variety of metabolic diseases. This is the first study that analyzes the association between erythrocyte CAT activity and candidate *CAT* SNPs with childhood obesity. The association study showed a significant positive association of the promoter variant −844A/G with childhood obesity and biomarkers of obesity such as weight, body mass index (BMI), BMI *Z*-Score, and adipocyte fatty acid-binding protein, along with a tendency toward significance with insulin resistance biomarkers. In addition, CAT erythrocyte activity was found to be significantly lower in obese children, and it was significantly correlated with obesity and insulin resistance biomarkers. No association was found between erythrocyte CAT activity and the SNP −844A/G. However, further *in vitro* and *in vivo* studies are needed to fully understand the role of CAT activity and SNPs in the development of insulin resistance in the setting of obesity. We hypothesize that CAT plays a role in early metabolic complications of obesity. *Antioxid. Redox Signal.* 00, 000–000.

Introduction

Obesity is a complex multifactorial disease that results from the interaction between an individual's environment and genetic background. Childhood obesity is becoming a major health concern in first-world countries and is nowadays a major risk factor for cardiovascular disease, type 2 diabetes, and metabolic syndrome in adulthood.

In recent years, oxidative stress has gained importance in the setting of obesity. Markers of oxidative stress have been associated with adult obesity, and it is known that oxidative damage and inflammation are present in children at the onset of obesity (2). The oxidative stress is also due to the alteration of the antioxidant defence system, which includes vitamins and antioxidant enzymes. One of the most important endogenous antioxidant enzymes is catalase (CAT), a peroxi-

Innovation

Previous studies have shown catalase (CAT) to be involved in metabolic diseases, including glucose disorders. The present study investigated the role of *CAT* genotypes and activity in prepubertal childhood obesity for the first time. Erythrocyte CAT activity was decreased in obese children and significantly correlated with insulin resistance biomarkers. Moreover, the single nucleotide polymorphism (SNP) −844A/G in the promoter of the *CAT* gene was associated with childhood obesity. Although the SNP −844A/G was not associated with CAT activity, it was associated with adipocyte fatty acid-binding protein levels, which could indicate a role of CAT in adipose tissue dysfunction present in obesity.

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somal antioxidant enzyme that degrades hydrogen peroxide into oxygen and water, which is known to be up-regulated upon oxidative stress. CAT activity has been found to be lower in nonobese children presenting insulin resistance defined as high values of homeostasis model assessment for insulin resistance (HOMA-IR) (8).

The study of single nucleotide polymorphisms (SNPs) could be an additional approach in the study of oxidative stress implications in obesity. Genes traditionally linked with obesity have been the most widely studied in the search for common genetic variations that may be implicated in the development of this disease. However, the studies including genes of antioxidant enzymes have been fewer. None of the previous studies has observed the association between antioxidant enzymes SNPs and obesity.

The *CAT* gene is located on chromosome 11p13 and contains 13 exons. To this moment, *CAT* SNPs have been related to a variety of diseases and conditions other than obesity; however, it is clear that they could play a role in glucose disorders (5). Interestingly, the A allele of the −844A/G polymorphism (rs769214) was associated with lower renutrition efficiency in the elderly, possibly through the creation of a PAX6-binding site on the *CAT* promoter that impacts its transcription, and subsequently, the processing of proglucagon and proinsulin (4). It has also been reported that the haplotype −844G; −89A; −20T in the 5′UTR region of the *CAT* gene is associated with a lower expression rate under oxidative stress damage as seen in a luciferase assay conducted in four human cell lines (9). These previous studies have proved that SNPs in the *CAT* gene may be a risk factor of metabolic diseases, but no studies have been conducted in order to study their association with the presence of obesity or its biomarkers.

Therefore, the aim of this study was to analyze the association of these known *CAT* SNPs along with representative SNPs from the whole *CAT* gene with childhood obesity in a cohort of prepubertal Spanish children. In addition, we wanted to analyze the main biomarkers related to obesity, insulin resistance, and oxidative stress and to study their association with erythrocyte CAT activity and SNPs in the setting of childhood obesity.

Anthropometric and biochemical characteristics of the study population

Characteristics of the study population are shown in Table 1. As expected, weight, body mass index (BMI), BMI *Z*-score, and waist circumference (WC) were significantly higher in obese children compared with the normal-weight controls.

The antioxidant defence status was affected in obese children, as CAT activity in erythrocytes as well as plasma total antioxidant capacity (TAC) were found to be significantly reduced in obese children. In addition, serum vitamin concentrations of α-tocopherol and β-carotene were lower in obese children; whereas no changes were observed in retinol. The oxidative stress biomarker oxidized low-density lipoprotein (ox-LDL) showed no differences between the two groups.

Insulin resistance and metabolic syndrome features showed the expected differences between obese and normal-weight children. Plasma adiponectin concentrations were significantly lower in obese children when compared with the normal-weight children, and oppositely leptin concentrations were higher.

Table 1. Anthropometric and Biochemical Characteristics of the Children

Variable	*Normal-weight*	*Obese*	p
Sex (male/female)	110/81	105/89	
Age (years)	8.9 ± 0.1	8.7 ± 0.1	0.127
Weight (kg)	29.2 ± 0.4	51.3 ± 0.9	< 0.001
BMI (kg/m^2)	16.60 ± 0.11	26.94 ± 0.26	< 0.001
BMI *Z*-score	−0.22 ± 0.04	3.57 ± 0.11	< 0.001
WC (cm)	58.3 ± 0.5	81.3 ± 0.9	< 0.001
SBP (mmHg)	95 ± 1	110 ± 1	< 0.001
DBP (mmHg)	58 ± 1	69 ± 1	< 0.001
Glucose (mg/dl)	83 ± 1	84 ± 1	0.846
Insulin (mU/L)	5.2 ± 0.2	10.3 ± 0.5	< 0.001
QUICKI	0.390 ± 0.002	0.351 ± 0.002	< 0.001
HOMA-IR	1.08 ± 0.04	2.14 ± 0.11	< 0.001
Adiponectin (mg/L)	27.87 ± 0.81	22.41 ± 0.79	< 0.001
Leptin (μg/L)	4.13 ± 0.29	22.69 ± 0.98	< 0.001
TAG (mg/dl)	54 ± 1	75 ± 3	< 0.001
Total cholesterol (mg/dl)	172 ± 2	165 ± 2	0.017
HDL-C (mg/dl)	65 ± 1	52 ± 1	< 0.001
LDL-C (mg/dl)	94 ± 2	96 ± 2	0.422
ApoA1 (mg/dl)	151 ± 2	133 ± 2	< 0.001
ApoB (mg/dl)	67 ± 1	72 ± 1	0.006
A-FABP (ng/ml)	9.60 ± 0.59	27.14 ± 1.45	< 0.001
ox-LDL (mg/L)	2.073 ± 0.152	1.912 ± 0.136	0.429
Catalase (K_{HB})	104 ± 3	90 ± 2	< 0.001
GR (μmol/g Hb/min)	3.624 ± 0.195	3.472 ± 0.123	0.507
GPX (mol/g Hb/min)	0.018 ± 0.001	0.019 ± 0.001	0.575
TAC (m*M* Trolox)	2.18 ± 0.05	1.76 ± 0.06	< 0.001
Retinol (μg/ml)	0.247 ± 0.005	0.256 ± 0.005	0.158
α-Tocopherol (μg/mL mg TAG)	0.162 ± 0.005	0.124 ± 0.004	< 0.001
β-carotene (μg/L mg TAG)	3.044 ± 0.192	1.358 ± 0.078	< 0.001
hsCRP (mg/L)	1.004 ± 0.283	3.546 ± 0.279	< 0.001

Data shown as mean ± SEM.

p, Student's *t*-test *p*-value.

A-FABP, adipocyte fatty acid-binding protein; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; DBP, diastolic blood pressure; GPX, glutathione peroxidase; GR, glutathione reductase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; hsCRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol; ox-LDL, oxidized LDL; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure; SEM, standard error of the means; TAC, total antioxidant capacity; TAG, triglycerides; WC, waist circumference.

Correlation of erythrocyte CAT activity with obesity, insulin resistance, and oxidative stress biomarkers in plasma

CAT activity in erythrocytes was found to be negatively correlated with obesity biomarkers as weight ($r = -0.120$, $p = 0.019$), BMI ($r = -0.160$, $p = 0.002$), and BMI *Z*-Score ($r = -0.168$, $p = 0.001$). In addition, CAT activity was correlated with insulin resistance biomarkers by showing a negative correlation with insulin ($r = -0.112$, $p = 0.031$) and HOMA-IR ($r = -0.104$, $p = 0.046$), and a positive correlation with quantitative insulin sensitivity check index (QUICKI) ($r = 0.110$,

$p = 0.035$). Moreover, CAT activity was negatively correlated with plasma leptin ($r = -0.221$, $p < 0.0001$), a biomarker that is intimately related with obesity and insulin resistance. With regard to oxidative stress biomarkers, CAT activity was found to be positively correlated with plasma β-carotene values ($r = 0.112$, $p = 0.030$), but not with retinol, α-tocopherol, or TAC. No significant correlation was found between erythrocyte CAT activity and plasma adipocyte fatty acid-binding protein (A-FABP).

These significant correlations between erythrocyte CAT activity and obesity, insulin resistance, and oxidative stress biomarkers show that an altered antioxidant defence status, including diminished CAT activity, could be involved in obesity and its complications as early as in infancy. Moreover, previous findings of reduced CAT activity in children with insulin resistance (8) are in line with these results, further supporting a possible role of oxidative stress in the development of insulin resistance associated with obesity.

Association of CAT SNPs with childhood obesity

We found the SNP −844G (rs769214) that was previously associated with higher renutrition efficiency in the elderly (4) to be associated with a higher risk of childhood obesity in our study population. In addition, two neighbour polymorphisms, −89A (rs7943316) and −20T (rs1049982), which form a haplotype with −844G, showed a highly similar association due to strong linkage disequilibrium (LD) between the variants ($D' = 1.00$; $r^2 = 1.00$) (Fig. 1). Population stratification was discarded by conducting a meta-analysis with the individual association results from each of the studied populations (Córdoba and Santiago de Compostela) ($I^2 = 0.00$). The results from the logistic regression analysis, adjusted for sex and age, as well as SNPs' allele frequencies and Hardy–Weinberg (HW) p-values are shown in Table 2. The following analyses were conducted considering the SNP −844A/G and not the haplotype, because the individual association results were stronger than the haplotype association results (data not shown).

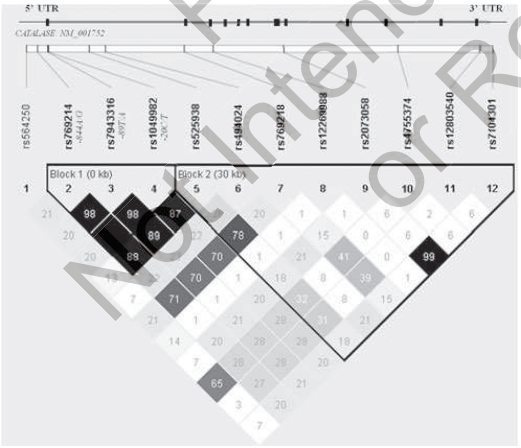

FIG. 1. Linkage disequilibrium diagram of the studied variants with the exon–intron scheme of the catalase gene. The r^2 values between the genotyped single nucleotide polymorphisms are shown in each cell, black cells correspond to $r^2 = 1.00$. Block definition followed the four gamete rule.

Association of the SNP −844A/G with obesity and insulin resistance biomarkers

The association of the SNP −844A/G with some of the analyzed biomarkers is shown in Figure 2. The presence of one risk allele was linked to an increase of 2.9 kg in weight or 0.43 U of BMI Z-Score in children. Insulin resistance markers showed a tendency toward significance, individuals with one risk allele had higher insulin (0.83 mU/L per risk allele, $p = 0.058$) and HOMA-IR values (0.17 U per risk allele, $p = 0.085$); whereas lower QUICKI (−0.01 units per risk allele, $p = 0.073$). This finding could indicate a role of *CAT* in the progress of insulin resistance and obesity in children.

Individuals carrying the variant −844G showed significantly higher plasma A-FABP levels, with an increase of 4.84 ng/ml per risk allele ($p = 0.013$). We hypothesize that this association could be due to the effect of this variant in adipose tissue. The haplotype −844G; −89A; −20T that we describe in the present study was previously shown to be associated to lower *CAT* expression in cell lines under high oxidative stress (9). As already described by previous studies (2), obese individuals show an enhanced oxidative stress in their adipose tissue. This stress could be observed as leading to a decrease of *CAT* expression in adipose tissue of the individuals carrying the risk haplotype in the present study. In addition, it is known that oxidative stress enhances adipocyte differentiation in adipose tissue, and that in this process the expression of A-FABP, a well-known adipose tissue marker, is enhanced (6). In this way, the decrease of CAT activity in adipose tissue would further increase the oxidative stress already present in obesity and alter the expression of A-FABP. The relationship between *CAT* genetic variations and circulating A-FABP further supports the link between the adipose tissue alterations and the development of obesity in the presence of an imbalanced redox state. Furthermore, the fact that the correlation between −844A/G and plasma A-FABP is not found to be paralleled by the correlation between erythrocyte CAT activity and A-FABP could be due to a depot-specific effect of the genotype at the initial stages of adipose tissue dysfunction in obesity.

No association was found between any of the studied SNPs and the antioxidant enzymes' activities (CAT, glutathione reductase [GR] and glutathione peroxidase [GPX]). Likewise, no association was found between the SNPs and the analyzed vitamins or TAC. The absence of association between −844A/G and CAT activity in erythrocytes does not differ from the previous studies conducted on this genetic variant, which found no association of erythrocyte CAT activity with the variant (4).

Conclusion

The importance of studying *CAT* genetic variants in metabolic diseases other than obesity has been highlighted in previous studies (5). In the present study, we show that CAT activity and its SNPs seem to play a role in various aspects of the metabolic alterations which take place in obesity. Here, we show for the first time that the previously studied *CAT* SNP −844A/G is associated with a higher childhood obesity risk along with increased weight, BMI, BMI Z-Score, and A-FABP values. In addition, in this study, we show for the first time

Table 2. Logistic Regression Analysis of *CAT* Single Nucleotide Polymorphisms with Obesity Under the Additive Model of Inheritance

SNP ID	Usual designation	Allele 1/ Allele 2	Obesity			Normal-weight			Risk allele	MAF		HW p	OR (95% CI)	p
			11	12	22	11	12	22		Obese	Normal-weight			
rs564250		G/A	117	53	7	117	59	5	A	0.299	0.326	0.864	1.00 (0.68, 1.46)	0.987
rs769214	**−844A/G**	A/G	65	89	23	84	81	16	G	0.503	0.448	0.414	**1.42 (1.03, 1.96)**	**0.035**
rs7943316	**−89T/A**	T/A	65	89	23	82	83	16	A	0.503	0.459	0.298	**1.38 (1.00, 1.91)**	**0.050**
rs1049982	**−20C/T**	C/T	65	89	23	83	81	16	T	0.503	0.450	0.414	**1.41 (1.02, 1.95)**	**0.039**
rs525938		A/G	73	84	20	86	81	14	G	0.475	0.448	0.400	1.29 (0.93, 1.79)	0.127
rs494024		G/A	87	70	20	68	89	24	A	0.396	0.492	0.727	0.73 (0.54, 1.00)	0.051
rs769218		G/A	86	77	14	102	68	11	A	0.435	0.376	0.790	1.30 (0.93, 1.83)	0.126
rs12269988		A/G	166	11	0	167	14	0	G	0.062	0.077	1.000	0.77 (0.34, 1.77)	0.542
rs2073058		A/G	85	78	14	91	79	11	G	0.441	0.437	0.354	1.11 (0.80, 1.56)	0.529
rs4755374		A/C	132	43	2	137	41	3	C	0.243	0.227	0.816	1.03 (0.66, 1.61)	0.892
rs12803540		A/G	124	46	5	139	36	4	G	0.263	0.201	0.381	1.35 (0.89, 2.07)	0.160
rs7104301	+33078A/G	A/G	84	79	14	91	79	11	G	0.446	0.437	0.202	1.13 (0.81, 1.59)	0.467

Values of the statistically significant associations are shown in bold.

Allele 1/2, major/minor allele; CI, confidence interval; HW, Hardy–Weinberg; MAF, minor allele frequency; OR, odds ratio (adjusted for sex and age); *p*, *p*-value of the logistic regression analysis; SNP, single nucleotide polymorphism.

that CAT activity in erythrocytes is decreased in obese children and it is significantly correlated with obesity, insulin resistance, and oxidative stress phenotypes.

Despite the previous associations found between SNP −844A/G and other disease states or phenotypes, the associations that we describe here need to be validated in larger cohorts of obese children. Furthermore, studies with human adipocyte models will be crucial to better understand the association of *CAT* polymorphisms with the development of insulin resistance and the possible alterations of adipose tissue, including a higher expression of markers such as A-FABP.

Notes

Study population

This is a case–control study in which we recruited 194 obese children (105 male and 89 female) and 191 normal-weight children (110 male and 81 female) of Caucasian ethnicity, aged 3–13, in two cities of Spain (Córdoba and Santiago de Compostela). Childhood obesity was defined according to Cole *et al.* (3). The inclusion criteria were prepubertal state, absence of disease related with nutritional status, and absence of endogenous obesity. The exclusion criteria were disease, undernutrition, pubertal stage, and the use of medication that alters blood pressure or glucose or lipid metabolism. After initial assessments at schools or primary care centres, children fulfilling the inclusion criteria were invited for a clinical examination in the appropriate participating hospital. The parents or guardians were informed about the purpose and procedures of the study before written consent was obtained, and all children gave their assent. Sex hormones were measured to confirm the prepubertal stage (data not shown). The protocol was performed in accordance with the Declaration of Helsinki (Edinburgh 2000, revised) and following the recommendations of

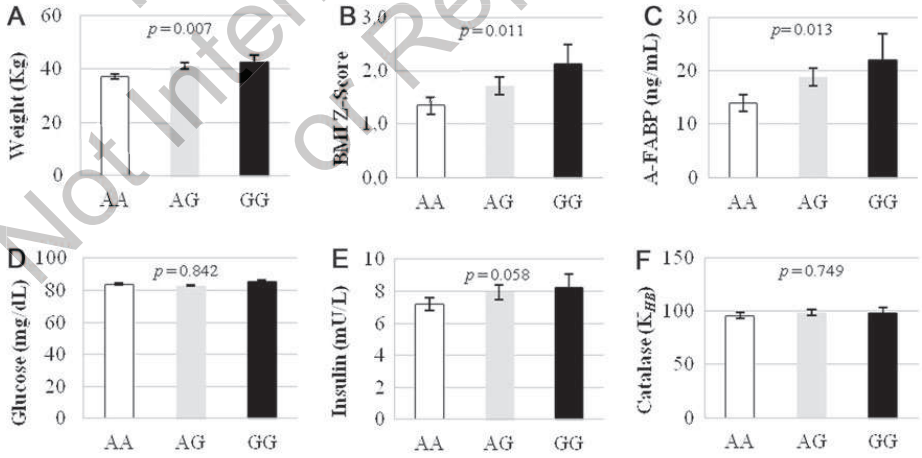

FIG. 2. Values of weight (A), body mass index (BMI) *Z*-Score (B), adipocyte fatty acid-binding protein (A-FABP) (C), glucose (D), insulin (E), and erythrocyte catalase (CAT) activity (F) of each genotype of the *CAT* gene variant −844A/G: AA (open), AG (gray), GG (solid). *p*, *p*-values from the linear regression association analysis adjusted for sex and age.

the Good Clinical Practice of the CEE (Document 111/3976/88 July 1990) and the legal in-force Spanish regulation that regulates Clinical Investigation in human beings (RD 223/04 about Clinical Assays) and was approved by the Ethics Committee on Human Research of the University of Granada, the Ethics Committee of the Reina Sofía University Hospital of Córdoba, and the Bioethics Committee of the University of Santiago de Compostela.

Anthropometric and biochemical measurements

Anthropometric measurements were taken by a single examiner with the children barefooted and in their underwear. Body weight (kg), height (cm), and WC (cm) were measured using standardized procedures, and BMI and BMI *Z*-Score were calculated. Obesity was defined according to BMI, using the age- and sex-specific cut-off points proposed by Cole *et al.* (linked to adult cut-offs of 25 and 30 kg/m^2) (3). Blood pressure was measured thrice by the same examiner following international recommendations. Blood samples were drawn *via* the antecubital vein after the children had fasted overnight.

Biochemical analyses were performed at the participating University Hospital Laboratories following internationally accepted quality control protocols. Specific biomarkers were analyzed using LINCOplexTM kits with human monoclonal antibodies (Linco Research). Adiponectin was measured using the kit catalogue # HADK1-61K-A, and leptin was analyzed using the kit catalogue # HADK2-61K-B. ox-LDL was quantified by using an enzyme-linked immunosorbent assay (ELISA) kit (Biomedica Medizinprodukte GmbH & Co KG). High-sensitivity C-reactive protein was determined with a particle-enhanced turbidimetric immunoassay. Plasma TAC was assessed by using the spectrophotometric commercial antioxidant assay kit from Cayman (Cat. No. 709001; Cayman). A-FABP plasma concentrations were measured by ELISA (Cat. No. RD191036200R; BioVendor). Erythrocyte activities of the antioxidant enzymes CAT and GR were assayed spectrophotometrically. Erythrocyte GPX activity was determined spectrophotometrically by the coupled enzyme procedure with tert-butyl hydroperoxide as substrate. Hemoglobin concentration in the blood samples was determined spectrophotometrically by the colorimetric cyanmethemoglobin method, using Sigma Diagnostic reagents. QUICKI and HOMA-IR were calculated using plasma glucose and insulin values. Retinol and α-tocopherol were analyzed by high-pressure liquid chromatography (HPLC) coupled to an electrochemical detector after extraction with 1-propanol. *β*-Carotene was also determined after extraction with 1-propanol in an HPLC system attached to a multi-wavelength ultraviolet detector set at 450 nm. All compounds were identified by predetermining the retention times of individual standards.

DNA isolation and genotyping

Genomic DNA was extracted using the QIAamp Blood kit (Qiagen). A total of 13 SNPs in the *CAT* gene were selected from the HapMap and NCBI databases based on their location. In addition to previously known SNPs, each missense variation was selected and then, others located in the promoter, 3′UTR and 5′UTR regions, all with a minor allele frequency higher than 0.05 in the Caucasian population. Genotyping was performed with the Illumina GoldenGate (Illumina) protocols on 96-well format Sentrix® arrays. Two hundred fifty nanograms of sample DNA were used per assay. Typing of the 13 SNPs resulted in genotype success rates of >95%, except for SNP rs1001179 that was excluded from further analysis. HW equilibrium for each SNP was examined by the exact test using PLINK version 1.07 software (7). The HW equilibrium *p*-values were greater than 0.05 in both obese and normal-weight groups for all SNPs, and the allele frequencies of the SNPs observed in the current study were similar to those reported in HapMap for Caucasians (data not shown). LD was analyzed with Haploview 4.2 software (1).

Statistical analysis

All statistical analyses were performed using either PLINK or SPSS (version 15.0.1). Normal distribution of clinical parameter data was assessed. Mean comparisons between obese and normal-weight children for continuous variables were performed using the Student's *t*-test for unpaired samples. Correlations were tested using the Pearson's correlation coefficient test.

The genotypic relative risk was assessed by comparing the obese group with the normal-weight group and calculating the odds ratio and the 95% confidence interval using logistic regression analysis under an additive model adjusted for age and sex. A meta-analysis was performed to discard population stratification from the two cities of recruitment. The association of the SNPs with obesity, insulin resistance, metabolic syndrome, and oxidative stress biomarkers was analyzed using a linear regression model adjusted for age and sex.

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References

1. Barrett JC, Fry B, Maller J, and Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263–265, 2005.
2. Codoñer-Franch P, Valls-Bellés V, Arilla-Codoñer A, and Alonso-Iglesias E. Oxidant mechanisms in childhood obesity: the link between inflammation and oxidative stress. *Transl Res* 158: 369–384, 2011.
3. Cole TJ, Bellizzi MC, Flegal KM, and Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 320: 1240–1243, 2000.
4. Hebert-Schuster M, Cottart CH, Laguillier-Morizot C, Raynaud-Simon A, Golmard JL, Cynober L, Beaudeux JL, Fabre EE, and Nivet-Antoine V. Catalase rs769214 SNP in

elderly malnutrition and during renutrition: is glucagon to blame? *Free Radic Biol Med* 51: 1583–1588, 2011.
5. Hebert-Schuster M, Fabre EE, and Nivet-Antoine V. Catalase polymorphisms and metabolic diseases. *Curr Opin Clin Nutr Metab Care* 15: 397–402, 2012.
6. Higuchi M, Dusting GJ, Peshavariya H, Jiang F, Hsiao ST, Chan EC, and Liu GS. Differentiation of human adipose-derived stem cells into fat involves reactive oxygen species and forkhead box o1 mediated upregulation of antioxidant enzymes. *Stem Cells Dev* 22: 878–888, 2013.
7. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, and Sham PC. PLINK: a toolset for whole-genome association and population-based linkage analysis. *Am J Hum Genet* 81: 559–575, 2007.
8. Shin MJ and Park E. Contribution of insulin resistance to reduced antioxidant enzymes and vitamins in nonobese Korean children. *Clin Chim Acta* 365: 200–205, 2006.
9. Wang Z, Li Y, Wang B, He Y, Wang Y, Xi H, Li Y, Wang Y, Zhu D, Jin J, Huang W, and Jin L. A haplotype of the catalase gene confers an increased risk of essential hypertension in Chinese Han. *Hum Mutat* 31: 272–278, 2010.

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Abbreviations Used

A-FABP = adipocyte fatty acid binding protein
ApoA1 = apolipoprotein A1
ApoB = apolipoprotein B
BMI = body mass index
CAT = catalase
CI = confidence interval
DBP = diastolic blood pressure
ELISA = enzyme-linked immunosorbent assay
GPX = glutathione peroxidise
GR = glutathione reductase
HDL-C = high-density lipoprotein cholesterol
HOMA-IR = homeostasis model assessment for insulin resistance
HPLC = high-pressure liquid chromatography
hsCRP = high-sensitivity C-reactive protein
HW = Hardy-Weinberg
LD = linkage disequilibrium
LDL-C = low-density lipoprotein cholesterol
MAF = minor allele frequency
OR = odds ratio
ox-LDL = oxidized LDL
QUICKI = quantitative insulin sensitivity check index
SBP = systolic blood pressure
SEM = standard error of the means
SNP = single nucleotide polymorphism
TAC = total antioxidant capacity
TAG = triglycerides
WC = waist circumference

Conclusions

Conclusions

1 Obese prepubertal Spanish children show a significantly altered profile of adipokines and biomarkers of obesity, insulin resistance, lipid metabolism, inflammation and endothelial damage when compared to the normal-weight group.

2 Obese prepubertal Spanish children show significant lower erythrocyte catalase activity as well as lower total antioxidant capacity, β-carotene and α-tocopherol in plasma when compared to their normal-weight counterparts.

3 The variant rs854566 in paraoxonase 1 gene is associated with lower obesity risk and higher lactonase, arylesterase and diazoxonase activities and lower paraoxonase activity of serum paraoxonase 1 in prepubertal children.

4 Variants from the catalase gene promoter rs769214, rs7693316 and rs1049982 are associated with higher childhood obesity risk and with higher body mass index, body mass index *z*-score, weight and fatty acid-binding protein 4.

5 A series of polymorphisms for glutathione peroxidases 4, 5 and 6, aldehyde oxidase 1, peroxiredoxin 5, oxidation resistance 1, oxidative stress responsive 1 and sirtuin 2 genes were also observed to be associated with childhood obesity and its phenotypes.

General conclusion

We found single nucleotide polymorphisms in the antioxidant defense system genes paraoxonase 1, catalase, glutathione peroxidases 4, 5 and 6, aldehyde oxidase 1, peroxiredoxin 5, oxidation resistance 1, oxidative stress responsive 1 and sirtuin 2 which were associated with obesity in prepubertal Spanish children. This finding supports the role of oxidative stress in the pathogenesis of obesity and its derived metabolic complications.

CONCLUSIONES

Conclusiones

1 Los niños obesos prepúberes españoles muestran un perfil alterado de adipoquinas y biomarcadores de obesidad, resistencia a la insulina, metabolismo lipídico, inflamación y daño endotelial comparados con el grupo normopeso.

2 Los niños obesos prepúberes españoles muestran menor actividad catalasa en eritrocitos y menor capacidad antioxidante total, β-carotenos y α-tocoferol en plasma comparados con los niños controles.

3 La variante rs854566 del gen paraoxonasa 1 se asocia a un menor riesgo de obesidad y a mayor actividad lactonasa, arilesterasa y diazoxonasa y a menor actividad paraoxonasa de la paraoxonasa 1 en suero en niños prepúberes.

4 Las variantes génicas del promotor del gen catalasa rs769214, rs7693316 y rs1049982 se asocian a un mayor riesgo de obesidad y a mayor índice de masa corporal, *z-score* del índice de masa corporal, peso y proteína de union a ácidos grasos 4.

5 Una serie de polimorfismos localizados en genes de glutatión peroxidasas 4, 5 y 6, aldehído oxidasa 1, peroxiredoxina 5, resistencia a la oxidación 1, respuesta a estrés oxidativo 1 y sirtuína 2 también se mostraron asociados a la obesidad infantil y sus fenotipos.

Conclusión general

Encontramos una serie de polimorfismos de un solo nucleótido en los genes relacionados con el sistema de defensa antioxidante paraoxonasa 1, catalasa, glutatión peroxidasas 4, 5 y 6, aldehído oxidasa 1, peroxiredoxina 5, resistencia a la oxidación 1, respuesta a estrés oxidativo 1 y sirtuína 2 asocidados a la obesidad en niños españoles prepúberes. Este hecho apoya el papel que juega el estrés oxidativo en la patogénesis de la obesidad y sus complicaciones metabólicas.

REFERENCES

REFERENCES

Aebi H. Catalase in vitro. Methods Enzymol 1984; 105: 121-126.

Ardlie KG, Kruglyak L, Seielstad M. Patterns of linkage disequilibrium in the human genome. Nat Rev Genet 2002; 3: 299-309.

Bondia-Pons I, Ryan L and Martinez JA. Oxidative stress and inflammation interactions in human obesity. J Physiol Biochem 2012; 68: 701-711.

Baráth A, Németh I, Karg E, Endreffy E, Bereczki C, Gellén B, Haszon I, Túri S. Roles of paraoxonase and oxidative stress in adolescents with uraemic, essential or obesity-induced hypertension. Kidney Blood Press Res 2006; 29: 144-151.

Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985; 113: 484-490.

Chen W, Yazicioglu M, Cobb MH. Characterization of OSR1, a Member of the Mammalian Ste20p/Germinal Center Kinase Subfamily. J Biol Chem 2004; 279: 11129-11136.

Codoñer-Franch P, Boix-García L, Simó-Jordá R, Del Castillo-Villaescusa C, Maset-Maldonado J, Valls-Bellés V. Is obesity associated with oxidative stress in children? Int J Pediatr Obes 2010; 5: 56–63.

Codoñer-Franch P, Valls-Bellés V, Arilla-Codoñer A, Alonso-Iglesias E. Oxidant mechanisms in childhood obesity: the link between inflammation and oxidative stress. Transl Res 2011; 158: 369-384.

Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: International survey. BMJ 2000; 320: 1240-1243.

Cook S, Weitzman M, Auinger P, Nguyen M, Dietz WH. Prevalence of metabolic syndrome phenotype in adolescents: findings from the third National Health and Nutrition Examination Survery. Arch Pediatr Adolesc Med 2003; 157:821-827.

Cruz ML, Weigensberg MJ, Huang TT, Ball G, Shaibi GQ, Goran MI. The metabolic syndrome in overweight Hispanic youth and the role of insulin sensitivity. J Clin Endocrinol Metab 2004; 89: 108-113.

Da Costa, L.A., Badawi, A. and El-Sohemy, A. Nutrigenetics and modulation of oxidative stress. Ann. Nutr. Metab 2012; 60: 27-36.

De Ferranti SD, Gauvreau K, Ludwig DS, Neufeld EJ, Newburger JW, Rifai N. Prevalence of the metabolic syndrome in American adolescents: findings from the Third National Health and Nutrition Examination Survey. Circulation 2004; 110: 2494-2497.

Drabkin DL. The standardization of hemoglobin measurement. Am J Med Sci 1948; 215: 110.

Draganov DI, Stetson PL, Watson CE, Billecke SS, La Du BN. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. J Biol Chem 2000; 275: 33435–33442.

Dubois L, Ohm Kyvik K, Girard M, Tatone-Tokuda F, Pérusse D, Hjelmborg J, Skytthe A, Rasmussen F, Wright MJ, Lichtenstein P, Martin NG. Genetic and environmental contributions to weight, height, and BMI from birth to 19 years of age: an international study of over 12,000 twin pairs. PLoS One 2012; 7: e30153.

Ebbeling CB, Pawlak DB, Ludwig DS. Childhood obesity: public-health crisis, common sense cure. Lancet 2002; 360: 473-482.

El-Koofy NM, El-Karaksy HM, Mandour IM, Anwar GM, El-Raziky MS, El-Hennawy AM. Genetic polymorphisms in non-alcoholic fatty liver disease in obese Egyptian children. Saudi J Gastroenterol 2011; 17: 265-270.

Elks CE, den Hoed M, Zhao JH, Sharp SJ, Wareham NJ, Loos RJ, Ong KK. Variability in the heritability of body mass index: a systematic review and meta-regression. Front Endocrinol 2012; 3: 29.

Fall T and Ingelsson E. Genome-wide association studies of obesity and metabolic syndrome. Mol Cell Endocrinol 2012; doi: 10.1016/j.mce.2012.08.018.

Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González A, Esquivel-Chirino C, Durante-Montiel I, Sánchez-Rivera G, Valadez-Vega C, Morales-González JA. Inflammation, Oxidative Stress, and Obesity. Int J Mol Sci 2011; 12: 3117-3132.

Flohé L, Günzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984; 105: 114-121.

Ford ES, Li C. Defining the metabolic syndrome in children and adolescents: Will the real definition please stand up? J Pediatr 2008; 152: 160-164.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M and Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004; 114: 1752-1761.

Garattini E, Fratelli M, Terao M. The mammalian aldehyde oxidase gene family. Hum Genomics 2009; 4: 119-130.

Garcia-Rodriguez CE, Mesa MD, Olza J, et al. Does consumption of two portions of salmon per week enhance the antioxidant defense system in pregnant women? Antioxid Redox Signal 2012; 16: 1401–1406.

Gupta N, Goel K, Shah P, Misra A. Childhood obesity in developing countries. Epidemiology, determinants, and prevention. Endocr Rev 2012; 33: 48-70.

Han JC, Lawlor DA, Kimm SY. Childhood obesity. Lancet 2010; 375: 1737-48.

Hardy J, Singleton A. Genomewide association studies and human disease. N Engl J Med 2009; 360: 1759-1768.

Hartmann T, Terao M, Garattini E, Teutloff C, Alfaro JF, Jones JP, Leimkühler S. The impact of single nucleotide polymorphisms on human aldehyde oxidase. Drug Metab Dispos 2011; 40: 856-864.

Hernández AF, Gómez MA, Pena G, Gil F, Rodrigo L, Villanueva E, Pla A. Effect of longterm exposure to pesticides on plasma esterases from plastic greenhouse workers. J Toxicol Environ Health A 2004; 67: 1095–1108.

Hinney A, Vogel CI, Hebebrand J. From monogenic to polygenic obesity: recent advances. Eur Child Adolesc Psychiatry 2010; 19: 297–310.

Hotamisligil GS. Inflammation and metabolic disorders. Nature 2006; 444: 860-867.

Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature. 2006; 440: 944-948.

Jing E, Gesta S, Kahn CR. Sirt2 Regulates Adipocyte Differentiation Involving FoxO1 Acetylation/Deacetylation. Cell Metab 2007; 6: 105-114.

Jorgensen TJ, Ruczinski I, Kessing B, Smith MW, Shugart YY, Alberg AJ. Hypothesis-driven candidate gene association studies: practical design and analytical considerations. Am J Epidemiol 2009; 170: 986-993.

Junge W, Klees H 1984 Arylesterase. In: Bergmeyer HU, Bergmeyer J, Graßi M, eds. Methods of enzymatic analysis, vol. IV. Weinheim: Verlag Chemie; 8–14

Katz A, Nambi SS, Mather K, Baron AD, Follman DA, Sullivan G, et al. Quantitative insulin sensitivity check index: A simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 2000; 85: 2402-2410.

Lyon HN, Hirshhorn JN. Genetics of common forms of obesity: a brief overview. Am J Clin Nutr 2005; 82(suppl): 215S–217S.

Manolio TA. Genome wide association studies and assesment of the risk of disease. N Engl J Med 2010; 363: 166-176.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28: 412-419.

Mayo O. A century of Hardy-Weinberg Equilibrium. Twin Res Hum Genet 2008; 11: 249-266.

McCarthy MI. Genomics, type 2 diabetes, and obesity. N Engl J Med 2010; 363: 2339-2350.

McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244: 6049-6055.

Nelson RA, Bremer AA. Insulin resistance and metabolic syndrome in the pediatric population. Metab Syndr Relat Disord 2010; 8: 1-14.

Olza J "Analisis de polimorfismos de genes asociados al sindrome metabolico en niños obesos". Tesis Doctoral inédita. Universidad de Granada 2011.

Olza J, Gil-Campos M, Leis R, Bueno G, Aguilera CM, Valle M, Cañete R, Tojo R, Moreno LA, Gil A. Presence of the metabolic syndrome in obese children at prepubertal age. Ann Nutr Metab 2011; 58: 343-350.

Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A & Aguilera CM. A gene variant of 11β-hydroxysteroid dehydrogenase type 1 is associated with obesity in children. Int J Obes 2012; 36: 1558-1563. (a)

Olza J, Aguilera CM, Gil-Campos M, Leis R, Bueno G, Martínez-Jiménez MD, Valle M, Cañete R, Tojo R, Moreno LA, Gil A. Myeloperoxidase is an early biomarker of inflammation and cardiovascular risk in prepubertal obese children. Diabetes Care 2012; 35: 2373-2376. (b)

Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Influence of variants in the NPY gene on obesity and metabolic syndrome features in Spanish children. Peptides 2013; 45: 22-27.

Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol 2011; 11: 85-97.

Pérez-Farinós N, López-Sobaler AM, Dal Re MÁ, Villar C, Labrado E, Robledo T, Ortega RM. The ALADINO Study: A National Study of Prevalence of Overweight and Obesity in Spanish Children in 2011. Biomed Res Int 2013; doi: 10.1155/2013/163687.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, Maller J, Sklar P, de Bakker PIW, Daly MJ, Sham PC. PLINK: a toolset for whole-genome association and population-based linkage analysis. Am J Hum Genet 2007; 81: 559-575

Rankinen T, Zuberi A, Chagnon YC, Weisnagel SJ, Argyropoulos G, Walts B, Pérusse L, Bouchard C. The human obesity gene map: the 2005 update. Obesity (Silver Spring) 2006; 14: 529-644.

Richter RJ, Furlong CE Determination of paraoxonase (PON1) status requires more than genotyping. Pharmacogenetics 1999; 9: 745-753.

Rolland-Cachera MF. Childhood obesity: current definitions and recommendations for their use. Int J Pediatr Obes 2011; 6: 325-331.

Rupérez AI, López-Guarnido O, Gil F, Olza J, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A, Aguilera CM. Paraoxonase 1 activity and genetic variation in childhood obesity. British Journal of Nutrition 2013; 110: 1639-1647. (a)

Rupérez AI, Olza J, Gil-Campos M, Leis R, Mesa MD, Tojo R, Cañete R, Gil A, Aguilera CM. Are Catalase -844A/G Polymorphism and Activity Associated with Childhood Obesity? Antioxidants & Redox Signaling 2013; doi: 10.1089/ars.2013.5386. (b)

Sobradillo B, Aguirre A, Aresti U, Bilbao A, Fernández Ramos C, Lizárraga A, et al. Curvas y tablas de Crecimiento y Desarrollo (Estudio Longitudinal y Transversal). 2004; Instituto de Investigacion Sobre Crecimiento Y Desarrollo. Fundacion Faustino Orbegozo Eizaguirre. María Díaz de Haro, 10 bis. 48013 Bilbao.

Stram DO. Tag SNP selection for association studies. Genet Epidemiol 2004; 27: 365-374.

Tanner JM. Growth at Adolescence: with a General Consideration of the Effects of Hereditary and Environmental Factors upon Growth and maturation from Birth to Maturity, 2en ed. Oxford, UK: Blackwell Scientific, 1962.

Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature 2006; 444: 875-880.

Vimaleswaran KS, Tachmazidou I, Zhao JH, Hirschhorn JN, Dudbridge F, Loos RJ. Candidate genes for obesity-susceptibility show enriched association within a large genome-wide association study for BMI. Hum Mol Genet 2012; 21: 4537-4542.

Viner RM, Segal TY, Lichtarowicz-Krynska E, hidmarsh P. Prevalence of the insulin resistance syndrome in obesity. Arch Dis Child 2005; 90: 10-14.

Wang K, Li WD, Zhang CK, Wang Z, Glessner JT, Grant SF, Zhao H, Hakonarson H, Price RA. A genome-wide association study on obesity and obesity-related traits. PLoS One 2011; 6: e18939.

Weiss R, Dziura J, Burgert T, Tanborlane W, Taksali S, Yeckel C, Allen K, Lopes M, Savoye M, Morrison J, Sherwin R, Caprio S. Obesity and metabolic syndrome in children and adolescents. N Engl J Med 2004; 350: 2362-2374.

Wheeler E, Huang N, Bochukova EG, Keogh JM, Lindsay S, Garg S, Henning E, Blackburn H, Loos RJ, Wareham NJ, O'Rahilly S, Hurles ME, Barroso I, Farooqi IS. Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity. Nat Genet 2013; 45: 513-517.

Wood ZA, Schröder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. Trends Biochem Sci 2003; 28: 32-40.

Xia Q, Grant SF. The genetics of human obesity. Ann N Y Acad Sci 2013; 1281: 178-190.

Yu Z, Han S, Cao X, Zhu C, Wang X, Guo X. Genetic polymorphisms in adipokine genes and the risk of obesity: a systematic review and meta-analysis. Obesity (Silver Spring) 2012; 20: 396-406.

Zanon-Moreno V, Asensio-Marquez EM, Ciancotti-Oliver L, Garcia-Medina JJ, Sanz P, Ortega-Azorin C, Pinazo-Duran MD, Ordovas JM, Corella D. Effects of polymorphisms in vitamin E, vitamin C, and glutathione peroxidase-related genes on serum biomarkers and associations with glaucoma. Mol Vis 2013; 19: 231-242.

ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
ADS	antioxidant defense system
ALT	alanine aminotransferase
ANGPTL2	angiopoietin-like 2
AOX1	aldehyde oxidase 1
ApoA-I	apolipoprotein A-I
ApoB	apolipoprotein B
ARE	antioxidant response element
AST	aspartate aminotransferase
BMI	body mass index
BP	blood pressure
CAT	catalase
CCL2	chemokine (C-C motif) ligand 2
CDDO-Im	2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide
CEBPβ	CCAAT/enhancer-binding protein beta
CHR	chromosome
CI	confidence interval
CNV	copy number variant
CXCL5	chemokine (C-X-C motif) ligand 5
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FABP-4	fatty acid-binding protein 4
FFA	free fatty acid
FGF21	fibroblast growth factor 21
FTO	fat mass and obesity
GGT	gamma-glutamil transferase
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GWAS	genome-wide association studies
Hb	hemoglobin
HDL	high-density lipoprotein

HDL-C	high-density lipoprotein cholesterol
HFD	high-fat diet
HOMA-IR	homeostasis model assessment for insulin resistance
HPLC	high-pressure liquid chromatography
hsCRP	high-sensitivity C-reactive protein
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HW	Hardy-weinberg
IL-18	interleukin 18
IL-6	interleukin 6
IL-8	interleukin 8
IMHP	2-isopropyl-4-methyl-6-hydroxy pyrimidine
IMT	intima media thickness
KEAP1	Kelch-like ECH-associated protein 1
LD	linkage disequilibrium
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LEP	leptin
LEPR	leptin receptor
MAF	minor allele frequency
MC4R	melanocortin receptor
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MMP-9	matrix metallopeptidase 9
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NAMPT	nicotinamide phosphoribosyltransferase
NCBI	National Center for Biotechnology Information
NQO1	NADPH quinone oxidoreductase
NRF2	nuclear erythroid factor 2-like 2
OR	odds ratio
Ox-LDL	oxidized LDL
OXR1	oxidation resistance 1
OXSR1	oxidative stress responsive 1
PAI-1	plasminogen activator inhibitor 1
PGC1α	PPARγ coactivator-1α
POMC	propiomelanocortin

PON1	paraoxonase 1
PPARγ	peroxisome proliferator activated receptor gamma
PRDX5	peroxiredoxin 5
PUFAs	poly-unsaturated fatty acids
QUICKI	quantitative insulin sensitivity check index
RBP4	retinol binding protein 4
ROS	reactive oxygen species
SBP	systolic blood pressure
SEM	standard error of the mean
SFRP5	secreted frizzled-related protein 5
SIRT2	sirtuin 2
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
TAC	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances
t-BOOH	tert-butyl hydroperoxide
TG	triacylclycerols
TNF-α	tumor necrosis factor alpha
WC	waist circumference
WHO	World Health Organization

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II. Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Influence of variants in the NPY gene on obesity and metabolic syndrome features in Spanish children. ***Peptides*** 2013; 45: 22-27.

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ORIGINAL ARTICLE

A gene variant of 11β-hydroxysteroid dehydrogenase type 1 is associated with obesity in children

J Olza[1], M Gil-Campos[2], R Leis[3], AI Rupérez[1], R Tojo[3], R Cañete[2], A Gil[1] and CM Aguilera[1]

BACKGROUND: The 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme catalyses the regeneration of active cortisol from inert cortisone and plays a critical role in tissue-specific corticosteroid reactions; therefore, 11β-HSD1 is a key molecule associated with the development of obesity. Despite evidence for its role in obesity, no genetic polymorphisms have been significantly associated with the disease *per se*.
OBJECTIVE: The aim of this study was to evaluate whether *HSD11B1* gene variants, which have never been studied before, are associated with obesity and its related traits, as well as its relation to biomarkers of inflammation, liver damage and cardiovascular disease in a cohort of Spanish children.
DESIGN: We performed a prospective case–control study.
SUBJECTS: A total of 534 children were examined and classified as being obese ($n = 292$) or normal weight ($n = 242$). Anthropometric and biochemical measurements related to obesity, including inflammation, liver damage and cardiovascular disease, were determined. Genomic DNA was extracted and 10 *HSD11B1* gene single-nucleotide polymorphisms (SNPs) were genotyped.
RESULTS: A novel SNP, rs3753519, was strongly associated with obesity and this SNP was the only statistically significant *HSD11B1* gene SNP remaining after a Bonferroni correction (odds ratio = 1.97 for allelic effect, 95% confidence interval 1.23–3.16; $P = 0.004$ and Bonferroni corrected $P = 0.046$). In addition, this SNP was significantly and positively associated with increased body mass index (BMI), BMI *z*-score, weight, waist circumference, plasma γ-glutamyl transpeptidase and plasma active plasminogen activator inhibitor 1. The SNP was negatively associated with plasma adiponectin and cortisol after adjusting for sex and age. None of the inflammation biomarkers tested were associated with the risk allele.
CONCLUSION: These data, which link an *HSD11B1* genotype with both disease prevalence and its related phenotypes, strongly support a role for the rs3753519 polymorphism in the pathogenesis of pediatric-onset obesity.

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Keywords: 11β-hydroxysteroid dehydrogenase type 1; genetic polymorphisms; children

INTRODUCTION

Cortisol, which is the major glucocorticoid in humans, plays an important role in regulating fuel metabolism, energy partitioning and body fat distribution. In addition to the hypothalamic–pituitary–adrenal axis controlling cortisol levels in the blood, intracellular cortisol levels in tissues can be controlled by local enzymes. For example, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyses the regeneration of active cortisol from inert cortisone, thereby amplifying cortisol levels and glucocorticoid receptor activation in adipose tissue, the liver and other tissue types.[1] The 11β-HSD1 is controlled by complex tissue-specific regulation. There is evidence that 11β-HSD1 adjusts local cortisol concentrations independently of plasma cortisol concentrations and this enzyme may be involved in obesity and its related complications.[2–4] Decreased cortisol levels in the liver secondary to reduced 11β-HSD1 expression and activity have been identified;[5–7] in contrast, increased 11β-HSD1 mRNA levels and enzyme activity have been observed in adult adipose tissue[8–10] and in children.[11]

Controversy exists regarding whether there is a comparable increase in 11β-HSD1 mRNA levels in subcutaneous and omental fat.[3] The mechanism of this dysregulation in human obesity remains uncertain; however, studies in animals have demonstrated a role for 11β-HSD1 in obesity. Mice overexpressing 11β-HSD1 in adipocytes demonstrate increased levels of adipose tissue corticosterone (the active metabolite in mice), hyperphagia, greater weight gain (particularly when fed a high-fat diet), increased accumulation of visceral adipose tissue, insulin resistance and increased expression of lipoprotein lipase in omental fat.[12] Conversely, 11β-HSD1 inhibition ameliorates the metabolic consequences of obesity, increases insulin sensitivity and reduces blood glucose levels in obese and diabetic mice.[13–16]

Despite evidence for a role of 11β-HSD1 in obesity, *HSD11B1* gene polymorphisms significantly associated with the disease or its complications have yet to be identified. *HSD11B1* gene variants have been reported to be associated with type 2 diabetes[17] and hypertension[18] but not with obesity *per se* in adults. A weak association of the rs2236905 *HSD11B1* single-nucleotide polymorphism (SNP) with metabolic syndrome in a Japanese population was recently found.[19] There is also one study that has reported a positive association of the ins4436A SNP in the *HSD11B1* gene with body mass index (BMI) and insulin resistance

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Table 1. Description of the *HSD11B1* SNPs analysed and genotypic information of the Spanish children tested

	Position[a]	*MAF*	*Alleles (M/m)*	*HWE* P-*value*[b]	*Call rate (%)*
rs2235543	5′ upstream	0.124	C/T	0.4837	100
rs12565406	5′ upstream	0.062	G/T	1	99.8
rs10082248	5′ upstream	0.059	G/A	1	99.8
rs4844880	5′ upstream	0.140	T/A	1	100
rs846910	5′ upstream	0.043	G/A	1	99.6
rs3753519	5′ upstream	0.084	G/A	1	99.8
rs4844488	Intron 3	0.032	A/G	1	99.8
rs846906	Intron 3	0.137	C/T	0.1996	75.0
rs6672256	Intron 3	0.194	T/A	0.8143	99.6
rs9430012	Intron 3	0.196	G/C	0.4776	98.5

Abbreviations: Call rate, rate of successful genotyping; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency; SNP, single nucleotide polymorphism. [a]SNP position on gene variant 1 (NM_005525.2). [b]*P*-value of the control group.

in obese children.[20] Thus, the objective of the current study was to evaluate whether the unstudied variations in the *HSD11B1* gene are associated with obesity and features of metabolic syndrome in a cohort of Spanish children.

MATERIALS AND METHODS

Study design

This study was designed as a case–control multicentre study in children. We recruited 292 (149 male and 143 female) obese children and 242 (135 male and 107 female) normal-weight children. All participants were Caucasian, aged 6–15 years, from two cities in Spain (that is, Cordoba, located in the south, and Santiago de Compostela, located in the north) and from primary care centres and schools. Childhood obesity was defined according to Cole *et al.*[21] The inclusion criteria were (1) the absence of disease related to nutritional status and (2) the absence of endogenous obesity. The exclusion criteria were (1) the presence of disease or undernutrition and (2) the use of medication that alters blood pressure, glucose or lipid metabolism. After the assessments made during the first visit to a school or primary care centre, the parents of children fulfilling the inclusion criteria were invited to bring their children to the pediatric unit of their local hospital for a clinical examination. The parents or guardians were informed about the purpose and procedures of the study before written consent was obtained. All children also provided consent. The protocol was performed in accordance with the Declaration of Helsinki Principles (Edinburgh 2000, revised) and followed the recommendations of both the Good Clinical Practice of the CEE (Document 111/3976/88 July, 1990) and the legal in-force Spanish Regulation that Regulates Clinical Investigation in Human Beings (RD 223/04 about Clinical Assays). The protocol was also approved by the ethics committees at all participating institutions.

Anthropometric and biochemical measurements

Anthropometric measurements were recorded by a single examiner with the children barefoot and in their underwear. Body weight (kg), height (cm) and waist circumference (cm) were measured using standardised procedures, and the BMI was calculated as weight (kg) divided by the square of the height (m^2). Obesity was defined according to the BMI using the age- and sex-specific cutoff points proposed by Cole *et al.*[21] (linked to the adult cutoffs of 25 and 30 $kg\,m^{-2}$). Blood pressure was measured three times by the same examiner using a mercury sphygmomanometer and following international recommendations. Blood samples were drawn via the antecubital vein after the patient had fasted overnight. Biochemical analyses were performed at the participating University Hospital Laboratories following internationally accepted quality control protocols.[22]

Other cardiovascular risk and inflammatory biomarkers were analysed using three different LINCOplex kits with the appropriate human monoclonal antibodies (Linco Research, MO, USA) on a Luminex 200 System (Luminex Corporation, Austin, TX, USA). The concentrations of soluble intercellular adhesion molecule 1 (sICAM-1), soluble endothelial selectin (sE-selectin), myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9) and total plasminogen activator inhibitor 1 (PAI-1) were measured using the LINCOplex kit with the catalogue number HCVD1-67-AK. Adiponectin, resistin and active PAI-1 were measured using the LINCOplex kit with the catalogue number HADK1-61K-A. Finally, interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) were analysed using the LINCOplex kit with the catalogue number HADK2-61K-B. C-reactive protein (CRP) was determined with a particle-enhanced turbidimetric ultrasensitive immunoassay (Dade Behring Inc., Deerfield, IL, USA).

DNA isolation and genotyping

Genomic DNA was extracted from buffy coats using the QIAamp Blood kit (Qiagen, Valencia, CA, USA). A total of 10 SNPs in the *HSD11B1* gene were selected from the HapMap and NCBI (National Center for Biotechnology Information) databases. The inclusion of an SNP in this analysis was based on its location. We first selected every SNP resulting in a missense variation and then selected other SNPs located in the promoter, 3′ untranslated region and 5′ untranslated region with a minor allele frequency >0.05 in the Caucasian population and minimum pairwise linkage disequilibrium of $r^2=0.8$ for the selection of TagSNPs. *HSD11B1* is transcribed from two promoters, resulting in two different transcripts (variant one, mRNA NM_005525, and variant two, mRNA NM_181755). Table 1 depicts the gene position in variant one of the 10 SNPs studied.

Genotyping was performed using the Illumina GoldenGate protocol (Illumina, San Diego, CA, USA) in 96-well Sentrix arrays. Per assay, 250 ng of sample DNA was used. The genotyping of the 10 SNPs resulted in a genotype success rate of $>95\%$, except for rs846906 (75%), which was excluded from the downstream analyses. The Hardy–Weinberg equilibrium for each SNP was examined with Fisher's exact test using PLINK version 1.07 software (available at http://pngu.mgh.harvard.edu/~purcell/plink). The Hardy–Weinberg equilibrium *P*-values were >0.05 in the case and control subjects for all SNPs (Table 1). The allelic frequencies of the SNPs observed in this study were similar to those reported in the HapMap for Caucasians (data not shown).

Statistical analyses

All statistical analyses were performed using either PLINK or SPSS (Statistical Package for the Social Sciences, version 15.0.1, Chicago, IL, USA). All continuous variables were expressed as the mean $\pm$ s.e.m. The normal distribution of the clinical parameter data was assessed with the Kolmogorov–Smirnov test. The insulin, the homeostatic model assessment for insulin resistance (HOMA-IR), total cholesterol, MMP-9 and total PAI-1 values were logarithmically transformed to approximate normal distributions. The homogeneity of variances was estimated using the Levene's test. The continuous variables in the obese and normal-weight children were compared using Student's *t*-test for unpaired samples. The genotypic relative risk was assessed by comparing the obese with the control group and calculating the odds ratio and 95% confidence interval (95% CI) using

Table 2. Anthropometric and biochemical parameters of the children

	Normal weight	*Obese*	P-*value*
n	242	292	
Sex (M/F)	135/107	149/143	0.273
Age (years)	9.73 ± 0.2	9.43 ± 0.2	0.172
Weight (kg)	32.9 ± 0.7	55.9 ± 1.0	**<0.001**
Height (m)	1.37 ± 0.01	1.41 ± 0.01	**0.001**
BMI (kg m^{-2})	17.14 ± 0.13	27.56 ± 0.24	**<0.001**
BMI *z*-score	−0.17 ± 0.04	3.49 ± 0.08	**<0.001**
Waist circumference (cm)	60.2 ± 0.5	84.0 ± 0.9	**<0.001**
Systolic BP (mm Hg)	97 ± 1	111 ± 1	**<0.001**
Diastolic BP (mm Hg)	60 ± 1	69 ± 1	**<0.001**
Glucose (mg dl^{-1})	84 ± 0	85 ± 1	0.772
Insulin (mU l^{-1})	5.95 ± 0.23	11.46 ± 0.51	**<0.001**
HOMA-IR	1.26 ± 0.05	2.43 ± 0.12	**<0.001**
Triacylglycerols (mg dl^{-1})	55 ± 1	75 ± 2	**<0.001**
ApoA1 (mg dl^{-1})	150 ± 2	132 ± 1	**<0.001**
ApoB (mg dl^{-1})	67 ± 1	71 ± 1	0.008
Cholesterol (mg dl^{-1})	171 ± 2	165 ± 2	0.012
HDL-c (mg dl^{-1})	64 ± 1	51 ± 1	**<0.001**
LDL-c (mg dl^{-1})	94 ± 2	97 ± 1	0.144
Adiponectin (mg l^{-1})	28.28 ± 0.77	22.57 ± 0.66	**<0.001**
Resistin (μg l^{-1})	9.65 ± 0.33	11.71 ± 0.34	**<0.001**
Leptin (μg l^{-1})	4.30 ± 0.26	22.91 ± 0.86	**<0.001**
Cortisol (nmol l^{-1})	362.71 ± 9.75	286.70 ± 9.00	**<0.001**
Testosterone (μg l^{-1})	0.49 ± 0.09	0.37 ± 0.03	0.263
ALT (U l^{-1})	16.76 ± 0.56	20.85 ± 0.50	**<0.001**
AST (U l^{-1})	23.70 ± 0.48	21.23 ± 0.40	**<0.001**
GGT (U l^{-1})	8.39 ± 0.27	10.84 ± 0.29	**<0.001**

Abbreviations: ALT, alanine transaminase; Apo, apolipoprotein; AST, aspartate transaminase; BMI, body mass index; BP, blood pressure; F, female; GGT, γ-glutamyl transpeptidase; HDL-c, high-density lipoprotein-cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-c, low-density lipoprotein-cholesterol; M, male. The *P*-values <0.001 are shown in bold.

logistic regression analysis under the additive model implemented in PLINK after adjusting for age and sex. Additional association analyses were performed independently of the recruitment city, and the homogeneity of both populations was assessed by a meta-analysis using the PLINK software. Linear regressions for the entire population or for each case and control group were performed using an additive model to estimate the associations of each SNP with the phenotypic parameters related to obesity and biomarkers of inflammation, liver damage and cardiovascular disease.

RESULTS

Patient characteristics

Table 2 displays the clinical characteristics of both the obese and control subjects. Weight, height, BMI, BMI *z*-score and waist circumference were significantly higher in the obese compared with the normal-weight children. Other biomarkers related to metabolic syndrome were different between both groups. Systolic and diastolic blood pressure, plasma triacylglycerols, apolipoprotein B, insulin and HOMA-IR were higher in the obese children. The plasma total cholesterol, high-density lipoprotein-cholesterol and apolipoprotein A-1 were all lower in the normal-weight children. Fasting plasma glucose and low-density lipoprotein-cholesterol concentrations did not differ between the two groups. Fasting plasma concentrations of resistin and leptin were significantly higher in the obese than in the normal-weight subjects; in contrast, reduced adiponectin and cortisol levels were observed in the obese children. The liver enzymes γ-glutamyl transpeptidase (GGT) and alanine transaminase (ALT) concentrations were significantly higher in the obese children; however, aspartate transaminase (AST) levels were lower in the obese population.

Table 3. Cardiovascular and inflammation characteristics of the children

	Normal weight	*Obese*	P-*value*
CRP (mg l^{-1})	0.95 ± 0.23	3.43 ± 0.25	**<0.001**
IL-6 (ng l^{-1})	4.50 ± 0.53	6.95 ± 0.75	**0.012**
IL-8 (ng l^{-1})	1.57 ± 0.11	2.16 ± 0.15	**0.003**
TNF-α (ng l^{-1})	3.06 ± 0.11	4.01 ± 0.13	**<0.001**
MCP-1 (ng l^{-1})	110.5 ± 3.67	110.7 ± 3.50	0.965
MMP-9 (μg l^{-1})	79.99 ± 3.15	88.42 ± 3.90	0.636
MPO (μg l^{-1})	13.10 ± 1.16	21.51 ± 1.70	**<0.001**
sE-selectin (μg l^{-1})	22.93 ± 0.76	31.26 ± 1.05	**<0.001**
sICAM-1 (mg l^{-1})	0.153 ± 0.004	0.175 ± 0.005	**<0.001**
sVAM-1 (mg l^{-1})	1.14 ± 0.02	1.07 ± 0.02	**0.024**
Active PAI-1 (μg l^{-1})	5.16 ± 0.27	11.96 ± 0.57	**<0.001**
Total PAI-1 (μg l^{-1})	18.95 ± 0.85	27.23 ± 1.01	**<0.001**

Abbreviations: CRP, C-reactive protein; IL, interleukin; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; MPO, myeloperoxidase; PAI, plasminogen activator inhibitor; sE-selection, soluble endothelial selection; sICAM, soluble intercellular adhesion molecule; sVAM, soluble vascular adhesion molecule; TNF, tumour necrosis factor. The *P*-values <0.05 are shown in bold.

Cardiovascular risk and inflammatory biomarkers were different between the two groups (Table 3). The sICAM-1, sE-selection, MPO and active PAI-1 levels were all significantly higher in the obese compared with the normal-weight group. Similarly, plasma levels of CRP, IL-6, IL-8 and TNF-α were higher in the obese group; however, MCP-1 and MMP-9 levels were the same in both groups.

Association of *HSD11B1* gene SNPs with obesity

The results of the association analyses of the nine SNPs are displayed in Table 4. In our study, five of the nine SNPs were nominally associated with obesity in children after adjusting for age and sex using an additive model; however, rs3753519 was the SNP most strongly associated with obesity, and it was the only statistically significant after Bonferroni correction (odds ratio = 1.97 for allelic effect, 95% CI 1.23–3.16; $P = 0.004$ and Bonferroni corrected $P = 0.045$). A meta-analysis was performed by combining the results from the two recruitment cities. There was a high level of homogeneity between these two populations for rs3753519 (Q Cochrane's statistic, $P = 0.882$). A consistent association of this SNP with obesity was identified after the meta-analysis (odds ratio = 2.05, $P = 0.003$).

Association of rs3753519 with obesity-related traits

We further examined the associations of rs3753519 with obesity-related quantitative traits (Table 5). This SNP was significantly ($P < 0.05$) associated with increased BMI, BMI *z*-score, weight, waist circumference and decreased adiponectin and cortisol levels after adjusting for sex and age when linear regression analyses were performed on the entire population. Children carrying allele A had increases in their BMI, weight and waist circumference of 2.4 kg m^{-2} (95% CI 1.21–3.64), 5 kg (95% CI 1.93–8.15) and 5.1 cm (95% CI 0.36–9.92) per allele, respectively. In contrast, reductions in plasma adiponectin 2.62 mg l^{-1} (95% CI −5.15 to −0.09) and cortisol 42.8 nmol l^{-1} (95% CI −77.24 to −8.52) concentrations were found per risk allele. However, when linear regression analyses were performed separately per case or on the control children, only the association of the risk allele with BMI remained significant in the case group ($P = 0.010$) β 1.12 (95% CI 0.25–1.99) after adjusting for age and sex. Following a Bonferroni correction, only the associations of rs3753519 with weight, BMI

Table 4. Genotypic distribution of the *HSD11B1* SNPs and their association with obesity in children

SNP	*Allele 1/allele 2*	*Case*			*Control*			*Risk allele*	*Risk allele frequency*		*OR (95% CI)*	P-*value*	P *corr**
		11	*12*	*22*	*11*	*12*	*22*		*Case*	*Control*			
rs2235543	C/T	217	64	10	194	46	1	T	0.144	0.099	1.51 (1.03 - 2.18)	0.031	0.313
rs12565406	G/T	253	35	2	214	27	0	T	0.067	0.056	1.21 (0.73 - 2.01)	0.465	1
rs10082248	G/A	251	36	3	220	21	0	A	0.072	0.044	1.72 (1.01 - 2.95)	0.046	0.464
rs4844880	T/A	211	68	12	187	51	3	A	0.158	0.118	1.36 (0.96 - 1.93)	0.080	0.803
rs846910	G/A	262	27	1	223	17	0	A	0.050	0.035	1.44 (0.78 - 2.65)	0.249	1
rs3753519	**G/A**	**233**	**52**	**5**	**214**	**27**	**0**	**A**	**0.107**	**0.056**	**1.97 (1.23 - 3.16)**	**0.004**	**0.046**
rs4844488	A/G	269	21	0	228	13	0	G	0.036	0.027	1.33 (0.65 - 2.73)	0.432	1
rs6672256	T/A	172	109	9	168	65	7	A	0.219	0.165	1.48 (1.07 - 2.04)	0.017	0.176
rs9430012	G/C	170	109	9	166	62	8	C	0.221	0.165	1.48 (1.07 - 2.04)	0.018	0.181

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism. The OR for each SNP was adjusted for age and sex using the additive model. **P*-values after Bonferroni corrections. SNP associated with obesity after Bonferroni correction (*P*-values <0.05) are shown in bold.

Table 5. Association of rs3753519 with obesity-related traits in children

	AA	*AG*	*GG*	*β (95% CI)*	P-*value*	P *corr**
n	5	79	449			
Age (years)	8.7 ± 1.3	9.5 ± 0.3	9.6 ± 0.1	−0.01 (−0.07 to 0.06)	0.879	1
Height (m)	1.40 ± 0.08	1.39 ± 0.02	1.39 ± 0.01	−0.01 (−0.02 to 0.00)	0.118	1
Weight (kg)	59.24 ± 7.69	48.14 ± 2.15	44.79 ± 0.87	5.04 (1.93 to 8.14)	**0.001**	**0.019**
BMI (kg m^{-2})	30.67 ± 2.02	24.27 ± 0.73	22.49 ± 0.28	2.39 (1.21 to 3.65)	**<0.001**	**0.003**
BMI *z*-score	4.69 ± 0.87	2.30 ± 0.24	1.71 ± 0.10	0.77 (0.32 to 1.22)	**<0.001**	**0.008**
Waist circumference (cm)	92.4 ± 5.1	75.9 ± 2.0	72.4 ± 0.8	5.14 (0.37 to 9.92)	**0.035**	0.369
Systolic BP (mm Hg)	117 ± 7	104 ± 2	105 ± 1	0.95 (−2.21 to 4.12)	0.555	1
Diastolic BP (mm Hg)	71 ± 7	65 ± 2	64 ± 1	1.35 (−1.24 to 3.94)	0.306	1
Glucose (mg dl^{-1})	76 ± 5	84 ± 1	85 ± 0	−1.45 (−2.94 to 0.03)	0.056	0.559
Insulin (mU l^{-1})	12.56 ± 4.63	9.55 ± 0.80	8.81 ± 0.35	0.05 (−0.00 to 0.11)	0.068	0.507
HOMA-IR	2.47 ± 0.97	1.99 ± 0.17	1.88 ± 0.08	0.04 (−0.01 to 0.10)	0.141	1
Triacylglycerols (mg dl^{-1})	102 ± 26	64 ± 3	66 ± 2	2.17 (−4.66 to 9.00)	0.534	1
ApoA1 (mg dl^{-1})	127 ± 11	137 ± 3	141 ± 1	−4.30 (−10.13 to 1.53)	0.149	1
ApoB (mg dl^{-1})	77 ± 7	67 ± 2	69 ± 1	−1.51 (−5.10 to 2.79)	0.567	1
Cholesterol (mg dl^{-1})	159 ± 7	162 ± 3	169 ± 1	−0.02 (−0.03 to −0.00)	**0.045**	0.662
HDL-c (mg dl^{-1})	44 ± 3	57 ± 2	57 ± 1	−1.35 (−4.51 to 1.82)	0.405	1
LDL-c (mg dl^{-1})	94 ± 6	91 ± 3	96 ± 1	−4.14 (−9.49 to 1.22)	0.131	1
Testosterone (μg l^{-1})	0.36 ± 0.08	0.41 ± 0.10	0.43 ± 0.05	0.06 (−0.14 to 0.25)	0.585	1
Adiponectin (mg l^{-1})	17.68 ± 2.36	23.43 ± 1.30	25.57 ± 0.56	−2.63 (−5.16 to −0.10)	**0.042**	0.609
Resistin (μg l^{-1})	10.57 ± 3.06	10.77 ± 0.65	10.78 ± 0.27	−0.02 (−1.23 to 1.19)	0.973	1
Leptin (μg l^{-1})	22.15 ± 2.41	15.71 ± 1.55	14.19 ± 0.70	2.12 (−0.98 to 5.22)	0.181	1
Cortisol (nmol l^{-1})	279.9 ± 64.2	292.3 ± 14.7	326.5 ± 7.6	−42.88 (−77.24 to −8.52)	**0.015**	0.120

Abbreviations: Apo, apolipoprotein; BMI, body mass index; BP, blood pressure; CI, confidence interval; HDL-c, high-density lipoprotein-cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; LDL-c, low-density lipoprotein-cholesterol. The β-coefficients represent the change in the absolute trait values of each additional risk allele. General linear models were used to examine associations, adjusted for age and sex and assuming an additive effect. **P*-values after Bonferroni corrections. The *P*-values <0.05 are shown in bold.

and BMI *z*-score remained significant (Table 5). No significant association of this SNP was linked to any other quantitative trait of obesity in either the case or control groups.

Association of rs3753519 with biomarkers of inflammation, cardiovascular risk and liver damage

To investigate the effect of the risk allele A of rs3753519 on the plasma biomarkers of inflammation and other obesity complications, such as cardiovascular risk and non-alcoholic fatty liver disease, additional linear regression analyses were performed (Table 6). In the entire population, none of the inflammation biomarkers (IL-6, IL-8, TNF-α, MCP-1 and CRP) displayed an association with the risk allele after adjusting for sex and age, nor were any of the biomarkers significant when the case and control groups were analysed separately.

The liver enzyme GGT, which is a marker of non-alcoholic fatty liver disease, was significantly increased in the children carrying allele A (1.03 U l^{-1} (95% CI 0.03 - 2.02) $P=0.040$ per allele). In contrast, transaminase concentrations (ALT and AST) did not show any association with the risk allele. Among the analysed biomarkers for cardiovascular risk (sICAM-1, sE-selectin, MPO, MMP-9 and active and total PAI-1), only the concentration of active PAI-1 was associated with allele A in the studied population ($P=0.007$) β 2.44 μg l^{-1} (95% CI 0.25 - 1.99); however, this significance was not upheld after a Bonferroni correction (Table 6). These associations were not observed when the linear regression analyses were conducted separately on the control or case groups (data not shown).

DISCUSSION

In this study, we identified a strong association of the rs3753519 *HSD11B1* SNP with obesity in Spanish children. The risk of obesity in children carrying the minor allele, 'allele A', was nearly double

Table 6. Association of the *HSD11B1* SNP, rs3753519, with biomarkers of inflammation, cardiovascular risk and liver damage in children

	AA	*AG*	*GG*	*β (95% CI)*	P-*value*	P *corr**
n	5	79	449			
ALT ($U\,l^{-1}$)	20 ± 3	22 ± 1	22 ± 0	−6.68 (−2.57 to 1.22)	0.485	1
AST ($U\,l^{-1}$)	15 ± 2	19 ± 1	19 ± 0	−0.83 (−2.25 to 0.59)	0.254	1
GGT ($U\,l^{-1}$)	12 ± 3	11 ± 0	10 ± 0	1.03 (0.03 to 2.03)	**0.045**	0.551
CRP ($mg\,l^{-1}$)	3.93 ± 0.61	3.22 ± 0.58	2.11 ± 0.19	0.79 (−0.71 to 2.29)	0.302	0.257
IL-6 ($ng\,l^{-1}$)	2.05 ± 0.63	6.24 ± 1.48	5.88 ± 0.51	−0.21 (−2.55 to 2.13)	0.859	1
IL-8 ($ng\,l^{-1}$)	1.20 ± 0.54	1.98 ± 0.21	1.89 ± 0.11	−0.02 (−0.49 to 0.45)	0.933	1
TNF-α ($ng\,l^{-1}$)	3.61 ± 0.67	3.92 ± 0.27	3.51 ± 0.10	0.32 (−0.12 to 0.75)	0.156	1
MCP-1 ($ng\,l^{-1}$)	111.60 ± 15.01	116.44 ± 7.69	109.64 ± 2.70	5.20 (−7.25 to 17.64)	0.413	1
MMP-9 ($\mu g\,l^{-1}$)	92.26 ± 19.45	86.81 ± 7.73	83.79 ± 2.72	−0.00 (−0.06 to 0.06)	0.991	1
MPO ($\mu g\,l^{-1}$)	17.25 ± 7.07	20.57 ± 2.63	17.19 ± 1.19	2.48 (−2.85 to 7.81)	0.363	1
sE-selectin ($\mu g\,l^{-1}$)	30.40 ± 13.83	27.73 ± 1.98	27.18 ± 0.73	0.56 (−2.85 to 3.98)	0.746	1
sICAM-1 ($mg\,l^{-1}$)	0.178 ± 0.024	0.160 ± 0.007	0.165 ± 0.003	−0.00 (−0.02 to 0.01)	0.679	1
sVAM-1 ($mg\,l^{-1}$)	0.96 ± 0.20	1.07 ± 0.04	1.11 ± 0.02	−0.05 (−0.13 to 0.024)	0.182	1
Active PAI-1 ($\mu g\,l^{-1}$)	21.89 ± 8.48	9.83 ± 1.03	8.56 ± 0.38	2.44 (0.65 to 4.22)	**0.008**	0.062
Total PAI-1 ($\mu g\,l^{-1}$)	31.40 ± 6.33	24.52 ± 1.90	23.16 ± 0.80	0.04 (−0.03 to 0.11)	0.254	1

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CI, confidence interval; CRP, C-reactive protein; GGT, γ-glutamyl transpeptidase; IL, interleukin; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; MPO, myeloperoxidase; PAI, plasminogen activator inhibitor; sE-selection, soluble endothelial selection; sICAM, soluble intercellular adhesion molecule; sVAM, soluble vascular adhesion molecule; SNP, single-nucleotide polymorphism; TNF, tumour necrosis factor. The β-coefficients represent the change in absolute trait values of each additional risk allele. General linear models were used to examine associations, adjusting for age and sex, and with the assumption of an additive effect. **P*-values after Bonferroni correction. The *P*-values <0.05 are shown in bold.

versus non-carriers. Furthermore, after adjusting for sex and age, the presence of this variant in our cohort was associated with increased BMI, BMI *z*-score, weight and waist circumference and decreased plasma fasting adiponectin levels. These data, which link the *HSD11B1* genotype with both disease prevalence and its related phenotypes, strongly support the role of the *HSD11B1* gene in the pathogenesis of obesity.

Despite observing a significant association of the allelic risk presence with common obesity-related phenotypes (BMI, weight, waist circumference and adiponectin) in the entire population, we failed to show these associations when the analyses were performed separately in the control and case groups (except for BMI in the case group). This may have been because of the relatively small sample size and narrow range of the tested quantitative traits in both the case and control groups. Nevertheless, the robust effect of the rs3753519 *HSD11B1* variant in obesity was evident, demonstrating a significant association with BMI in the obese group despite the relatively small sample size. However, additional association studies with a larger independent Caucasian population are necessary to validate our current findings and investigate the associations of this SNP with obesity-related quantitative traits.

The regulation of *HSD11B1* expression is highly tissue specific and involves the use of two alternative promoters: a distal promoter, P1, and a proximal promoter, P2.[23] In a recent study, transcription from P2 predominated in human liver, lung and subcutaneous adipose tissues, whereas transcription from P1 predominated in human tumour cell lines.[24] rs3753519 is located in the P2 promoter exactly 2679 nucleotides 5′ to the transcriptional start codon; therefore, this SNP may affect the transcription rate of the *HSD11B1* gene. Although the functional consequences of this polymorphism were assessed using several different web tools for expression quantitative trait loci and transcription factor binding sites, there were no positive results (data not shown). Polymorphisms in the P2 promoter region (rs846910) and an intronic enhancer (rs12086634) have been associated with type 2 diabetes and/or hypertension in three different populations, although not with obesity *per se*.[17,18,25] The G allele of rs12086634 has been associated with lower 11β-HSD1 transcriptional activity *in vitro*;[26] however, no functional consequences of the allelic variation rs846910 have been found *in vitro*.[27] Another polymorphism in the P2 promoter located two nucleotides 5′ to the translation initiation site (rs13306421) has shown functional consequences on 11β-HSD1 enzyme activity *in vitro* by producing higher enzyme expression and activity levels;[27] however, the presence of this variant has not been detected in the Caucasian population, and its association with disease has not been published.

In the present study, the previously reported association of rs846910 with diabetes and hypertension-related phenotypes was not found, and its association with obesity was not significant despite being in linkage disequilibrium with the polymorphism rs3753519 ($D'=1$; $r^2=0.495$ calculated with Haploview 4.2 software available at www.broad.mit.edu/mpg/haploview/). The reason for this lack of a clear association between 11β-HSD1 genetic variability and obesity has yet to be reported. Despite evidence of its effect in animal and human obesity, this lack of association may be because neither of the SNPs investigated were functional, and the weak associations found with obesity-related phenotypes could be attributable to a linkage disequilibrium with a functional locus.

The reduction in plasma cortisol concentrations associated with the presence of rs3753519 risk allele A, which was identified in the present study, may indicate an effect of this variant on gene transcription and enzyme activity. Lower hepatic 11β-HSD1 activity (assessed by the urinary tetrahydrocortisol + 5α-tetrahydrocortisol/tetrahydrocortisone ratio) has been reported in obese children[28] and is supported by the finding that the activation of oral cortisone to cortisol is impaired in obesity.[5] However, there is no consensus on plasma cortisol levels in obese subjects. It should be noted that the interpretation of plasma cortisol values is complicated by cortisol being secreted episodically;[29] thus, a single spot value may be unrepresentative. This problem can be obviated by measuring the 24-h mean plasma cortisol concentrations or 24-h integrated plasma concentration.[30] Only studies using one of these parameters should be accepted for the critical evaluation of plasma cortisol concentrations in obesity. Decreased plasma cortisol concentrations have been found in obese individuals using these parameters, and this is likely because of the subnormal responsiveness to feedback stimulation in the hypothalamic–pituitary–adrenal axis.[30] Although the method of measuring plasma cortisol concentrations used in this study is a limitation, the plasma cortisol concentrations were analysed under the same conditions in all subjects (that is, in the

morning at 0800–0830 h after an overnight fast). Therefore, the decreased cortisol concentrations found in obese children could support this hypothesis.

Independent of plasma cortisol concentration, tissue glucocorticoid dysregulation has been described in common obesity. Decreased cortisol levels in the liver secondary to reduced 11β-HSD1 activity[5–7] and increased 11β-HSD1 mRNA levels and activity in adipose tissue have been reported.[6–10] However, the underlying mechanisms for the emerging tissue-specific expression patterns are poorly understood. Recently, it has been proposed that several key regulators of lipid metabolism and inflammation, including peroxisome proliferator-activated receptor α and γ, liver X receptor and CCAAT/enhancer-binding proteins, participate in regulating *HSD11B1* expression.[2] In this study, no associations between the *HSD11B1* variant and circulating lipids or inflammation biomarkers were identified. Therefore, although tissue concentrations should be assessed, these results could rule out this hypothesis, at least in children. The association of rs3753519 with increased concentrations of the liver enzyme GGT, which is a classical marker of fatty liver disease, as well as decreased concentrations of adiponectin, which is a marker of adipose tissue alteration, and increased concentrations of active PAI-1, a marker of cardiovascular disease, could imply a possible role for this variant in the aetiology of obesity. We conclude that the rs3753519 gene polymorphism in *HSD11B1* is associated with susceptibility to pediatric-onset obesity and its complications; however, this finding should be replicated in an independent cohort.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Stewart PM, Krozowski ZS. 11 beta-Hydroxysteroid dehydrogenase. *Vitam Horm* 1999; **57**: 249–324.
2 Staab CA, Maser E. 11beta-Hydroxysteroid dehydrogenase type 1 is an important regulator at the interface of obesity and inflammation. *J Steroid Biochem Mol Biol* 2010; **119**: 56–72.
3 Morton NM. Obesity and corticosteroids: 11beta-hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. *Mol Cell Endocrinol* 2010; **316**: 154–164.
4 Anagnostis P, Athyros VG, Tziomalos K, Karagiannis A, Mikhailidis DP. Clinical review: the pathogenetic role of cortisol in the metabolic syndrome: a hypothesis. *J Clin Endocrinol Metab* 2009; **94**: 2692–2701.
5 Stewart PM, Boulton A, Kumar S, Clark PMS, Shackleton CHL. Cortisol metabolism in human obesity: impaired cortisone--cortisol conversion in subjects with central adiposity. *J Clin Endocrinol Metab* 1999; **84**: 1022–1027.
6 Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DE, Johnson O *et al.* Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab* 2001; **86**: 1418–1421.
7 Simonyte K, Rask E, Näslund I, Angelhed JE, Lönn L, Olsson T *et al.* Obesity is accompanied by disturbances in peripheral glucocorticoid metabolism and changes in FA recycling. *Obesity* 2009; **17**: 1982–1987.
8 Sandeep TC, Andrew R, Homer NZ, Andrews RC, Smith K *et al.* Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11beta-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. *Diabetes* 2005; **54**: 872–879.
9 Paulmyer-Lacroix O, Boullu S, Oliver C, Alessi MC, Grino M. Expression of the mRNA coding for 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. *J Clin Endocrinol Metab* 2002; **87**: 2701–2705.
10 Paulsen SK, Pedersen SB, Fisker S, Richelsen B. 11Beta-HSD type 1 expression in human adipose tissue: impact of gender, obesity, and fat localization. *Obesity* 2007; **15**: 1954–1960.
11 Mericq V, Medina P, Kakarieka E, Márquez L, Johnson MC, Iñiguez G. Differences in expression and activity of 11beta-hydroxysteroid dehydrogenase type 1 and 2 in human placentas of term pregnancies according to birth weight and gender. *Eur J Endocrinol* 2009; **161**: 419–425.
12 Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR *et al.* A transgenic model of visceral obesity and the metabolic syndrome. *Science* 2001; **294**: 2166–2170.
13 Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P *et al.* 11-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity or stress. *Proc Natl Acad Sci USA* 1997; **94**: 14924–14929.
14 Morton NM, Holmes MC, Fiévet C, Staels B, Tailleux A, Mullins JJ *et al.* Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11-hydroxysteroid dehydrogenase type 1 null mice. *J Biol Chem* 2001; **276**: 41293–41300.
15 Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C *et al.* Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11-hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* 2004; **53**: 931–938.
16 Alberts P, Nilsson C, Selen G, Engblom LO, Edling NH, Norling S *et al.* Selective inhibition of 11-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* 2003; **144**: 4755–4762.
17 Nair S, Lee YH, Lindsay RS, Walker BR, Tataranni PA, Bogardus C *et al.* 11-Hydroxysteroid dehydrogenase type 1: genetic polymorphisms are associated with type 2 diabetes in Pima Indians independently of obesity and expression in adipocyte and muscle. *Diabetologia* 2004; **47**: 1088–1095.
18 Franks PW, Knowler WC, Nair S, Koska J, Lee YH, Lindsay RS *et al.* Interaction between an 11HSD1 gene variant and birth era modifies the risk of hypertension in Pima Indians. *Hypertension* 2004; **44**: 681–688.
19 Miyamoto Y, Morisaki H, Yamanaka I, Kokubo Y, Masuzaki H, Okayama A *et al.* Association study of 11beta-hydroxysteroid dehydrogenase type 1 gene polymorphisms and metabolic syndrome in urban Japanese cohort. *Diabetes Res Clin Pract* 2009; **85**: 32–38.
20 Gelernter-Yaniv L, Feng N, Sebring NG, Hochberg Z, Yanovski JA. Associations between a polymorphism in the 11 beta hydroxysteroid dehydrogenase type I gene and body composition. *Int J Obes Relat Metab Disord* 2003; **27**: 983–986.
21 Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 2000; **320**: 1240–1243.
22 Gil-Campos M, Ramírez Tortosa MC, Aguilera CM, Cañete R, Gil A. Fasting and postprandial adiponectin alterations anticipate NEFA and TNF-α changes in prepubertal obese children. *Nutr Metab Cardiovasc Dis* 2011; **21**: 62–68.
23 Bruley C, Lyons V, Worsley AG, Wilde MD, Darlington GD, Morton NM *et al.* A novel promoter for the 11- hydroxysteroid dehydrogenase type 1 gene is active in lung and is C/EBP independent. *Endocrinology* 2006; **147**: 2879–2885.
24 Staab CA, Stegk JP, Haenisch S, Neiß E, Köbsch K, Ebert B *et al.* Analysis of alternative promoter usage in expression of HSD11B1 including the development of a transcript-specific quantitative real-time PCR method. *Chem Biol Interact* 2011; **191**: 104–112.
25 Morales MA, Carvajal CA, Ortiz E, Mosso LM, Artigas RA, Owen GI *et al.* Possible pathogenetic role of 11-hydroxysteroid dehydrogenase type 1 (11HSD1) gene polymorphisms in arterial hypertension. *Rev Med Chil* 2008; **136**: 701–710.
26 Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W *et al.* Mutations in the genes encoding 11-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. *Nat Genet* 2003; **34**: 434–439.
27 Malavasi EL, Kelly V, Nath N, Gambineri A, Dakin RS, Pagotto U *et al.* Functional effects of polymorphisms in the human gene encoding 11 beta-hydroxysteroid dehydrogenase type 1 (11 beta-HSD1): a sequence variant at the translation start of 11 beta-HSD1 alters enzyme levels. *Endocrinology* 2010; **151**: 195–202.
28 Wiegand S, Richardt A, Remer T, Wudy SA, Tomlinson JW, Hughes B *et al.* Reduced 11beta-hydroxysteroid dehydrogenase type 1 activity in obese boys. *Eur J Endocrinol* 2007; **157**: 319–324.
29 Hellman L, Nakada F, Curti J, Weitzman ED, Kream J, Roffwarg H *et al.* Cortisol is secreted episodically by normal man. *J Clin Endocrinol Metab* 1970; **30**: 411–422.
30 Salehi M, Ferenczi A, Zumoff B. Obesity and cortisol status. *Horm Metab Res* 2005; **37**: 193–197.

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Influence of variants in the *NPY* gene on obesity and metabolic syndrome features in Spanish children

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ABSTRACT

Variants in the neuropeptide Y (*NPY*) gene have been associated with obesity and its traits. The objective of the present study was to evaluate the association of single nucleotide polymorphisms (SNPs) in the *NPY* gene with obesity, metabolic syndrome features, and inflammatory and cardiovascular disease (CVD) risk biomarkers in Spanish children. We recruited 292 obese children and 242 normal-body mass index (BMI) children. Height, weight, BMI, waist circumference, clinical and metabolic markers, adipokines, and inflammatory (PCR, IL-6, IL-8 and TNF-α) and CVD risk biomarkers (MPO, MMP-9, sE-selectin, sVCAM, sICAM, and PAI-1) were analyzed. Seven SNPs in the *NPY* gene were genotyped. The results of our study indicate that anthropometric measurements, clinical and metabolic markers, adipokines (leptin and resistin), and inflammatory and CVD risk biomarkers were generally elevated in the obese group. The exceptions to this finding included cholesterol, HDL-c, and adiponectin, which were lower in the obese group, and glucose, LDL-c, and MMP-9, which did not differ between the groups. Both rs16147 and rs16131 were associated with the risk of obesity, and the latter was also associated with insulin resistance, triacylglycerols, leptin, and HDL-c. Thus, we confirm the association of rs16147 with obesity, and we demonstrate for the first time the association of rs16131 with obesity and its possible impact on the early onset of metabolic syndrome features, mainly triacylglycerols, in children.

1. Introduction

Neuropeptide Y (NPY) is a peptide that acts as a neurotransmitter or neuromodulator. It has been implicated in several human diseases including obesity, alcoholism, schizophrenia, and depression, each of which might be considered to have behavioral or psychiatric components [3]. The role of NPY in the hypothalamic control of energy balance is well established. When this potent orexigenic neuropeptide is chronically administered to the central nervous system, it leads to an increase in food intake, body weight, and adiposity in rats [25,29]. The obesity induced by NPY is due not only to hyperphagia but also to increased accumulation of lipids in white adipose tissue, inhibition of thermogenesis in brown adipose tissue, stimulation of hyperinsulinemia, and hypersecretion of corticosteroids [1,21]. Although there is extensive evidence of the key role of NPY in energy regulation in rats, evidence in humans is limited.

The first studies showing a positive association of an *NPY* gene variant (-880I/D) with obesity were those performed by Bray et al. [2] in Mexican-American families. Additionally, there have been many studies examining the functional Leu7Pro polymorphism (rs16139). This SNP has been associated with a large number of conditions related to obesity and metabolic syndrome traits, including increased body mass index (BMI) in adults [6], development of obesity in young adults [28], risk of hypertension [11], high plasma

Abbreviations: NPY, neuropeptide Y; SNP, single nucleotide polymorphism; TAG, triacylglycerols; LDL-c, low-density lipoprotein-cholesterol; BP, blood pressure; BMI, body mass index; CVD, cardiovascular diseases; IOTF, International Obesity Task Force; PAI, 1 plasminogen activator inhibitor-1; IL, interleukin; TNF-α, tumor necrosis factor alpha; sICAM-1, soluble intercellular adhesion molecule 1; sE-selectin, soluble endothelial selectin; MPO, myeloperoxidase; MMP-9, matrix metalloproteinase 9; CRP, C reactive protein; MAF, minor allele frequency; LD, linkage disequilibrium; HOMA-IR, homeostasis model assessment for insulin resistance; OR, odds ratio; CI, confidence interval; VLDL, very low density lipoproteins.

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low-density lipoprotein-cholesterol (LDL-c) in children and adults [9,22], and elevated plasma triacylglycerols (TAG) [10]. This variant has been associated with metabolic syndrome in patients with coronary artery disease [15]. This SNP has also been shown to correlate with high birth body weight in preschoolers [10], the risk of an accelerated atherosclerotic process or carotid atherosclerosis in adults [11,17], and the risk of type 2 diabetes mellitus in adults [18,26].

Other studies of SNPs in the *NPY* gene have reported an association of rs16147 and rs16135 with ischemic stroke [12,13,31] and the onset of atherosclerosis [23]. Additionally, rs16147 has been associated with being overweight [8] and dietary fat intake and changes in blood pressure (BP) [32]. However, there are only two studies that have investigated the association of rs16147 with obesity; one study found no association of this variant with obesity in two different cohorts of adults [30], and the other demonstrated a significant effect of this variation on age-dependent body weight and BMI during childhood and adolescence in a German population [7]. No associations so far have been reported for rs16131. Thus, the objective of the present study was to evaluate whether some variants in the *NPY* gene, including rs16131, rs16139, and rs16147, are associated with obesity, metabolic syndrome features, and inflammatory and cardiovascular disease (CVD) risk biomarkers in a cohort of Spanish children.

2. Materials and methods

2.1. Study design

This is a case–control multicenter study in children. We recruited 292 (149 male and 143 female) obese children and 242 (135 male and 107 female) normal-BMI children, all of them of European-Caucasian heritage and aged 5–15 years between May 2007 and May 2010 in two cities in Spain (Cordoba and Santiago de Compostela). Childhood obesity was defined according to the International Obesity Task Force (IOTF) reference for children [5]. The inclusion criteria were European-Caucasian heritage and the absence of congenital metabolic diseases. The exclusion criteria were non-European-Caucasian heritage, the presence of congenital metabolic diseases (e.g., diabetes or hyperlipidemia), undernutrition, and the use of medication that alters BP, glucose or lipid metabolism. After the initial assessments were completed at the school or primary care center, the children fulfilling the inclusion criteria were invited for a clinical examination at the appropriate participating hospital. The parents or guardians were informed about the purpose and procedures of the study before written consent was obtained, and all children gave their assent. The study was compliant with the Declaration of Helsinki (Edinburgh 2 000 revised) and followed the recommendations of the Good Clinical Practice of the CEE (Document 111/3976/88 July 1990), and the legally enforced Spanish regulation, which regulates the clinical investigation of human beings (RD 223/04 about clinical trials). The Ethics Committee on Human Research of the University of Granada, the Ethics Committee of the Reina Sofía University Hospital of Cordoba, and the Bioethics Committee of the University of Santiago de Compostela approved the study.

2.2. Anthropometric and biochemical measurements

The anthropometric measurements were taken by a single examiner at each hospital. The children were barefoot and in their underwear when the measurements were taken. Body weight (kg) was measured using a standard beam balance. Height (cm) was measured using a precision stadiometer. BMI, defined as weight (kg) divided by the square of height (m^2), was calculated. Waist circumference was measured by applying an inelastic tape horizontally midway between the lowest rib margin and the iliac crest of the standing child at the end of a gentle expiration. BP was measured three times by the same examiner using a mercury sphygmomanometer and following international recommendations [16]. The blood samples were drawn via the antecubital vein after the patient had fasted overnight. The biochemical analyses were performed at the participating University Hospital Laboratories following internationally accepted quality control protocols.

2.3. Adipocytokines, inflammation, and CVD risk biomarkers

The adipocytokines and inflammatory and CVD risk biomarkers were analyzed using three different LINCOplex™ kits with human monoclonal antibodies (Linco Research, MO, USA) on a Luminex® 200™ System (Luminex Corporation, TX). The three kits were the following: (1) Adiponectin (CV: 7.9%), resistin (CV: 6.0%), and active plasminogen activator inhibitor-1 (PAI-1) (CV: 6.6%) (Cat. HADK1-61K-A); (2) interleukin (IL)-6 (CV: 7.8%), IL-8 (CV: 7.9%), leptin (CV: 7.9%), and tumor necrosis factor alpha (TNF-α) (CV: 7.8%) (Cat. HADK2-61K-B); and (3) soluble intercellular adhesion molecule-1 (sICAM-1) (CV: 7.9%), soluble endothelial selectin (sE-selectin) (CV: 11.2%), myeloperoxidase (MPO) (CV: 12.3%), matrix metalloproteinase-9 (MMP-9) (CV: 6.8%), and total PAI-1 (CV: 11.8%) (Cat. HCVD1-67AK). C reactive protein (CRP) (CV: 4%) levels were determined with a particle-enhanced turbidimetric immunoassay (Dade Behring Inc., Deerfield, IL).

2.4. DNA isolation and genotyping

Genomic DNA was extracted from buffy coats using the QIAamp Blood kit (Qiagen, Valencia CA, USA). A total of seven SNPs in different regions of the *NPY* gene were selected from the HapMap and NCBI databases and were among those with a minor allele frequency (MAF) higher than 0.05 and a minimum pair wise linkage disequilibrium (LD) of $r^2 = 0.8$ for the Caucasian population. Genotyping was performed with the Illumina GoldenGate (Illumina, San Diego, CA, USA) protocol on 96-well format Sentrix® arrays. Two hundred and fifty nanograms of DNA sample were used per assay.

The genotyped SNPs had genotype success rates of >95%, with the exception of rs16148 (84.5%), which was excluded from further analyses. The Hardy–Weinberg equilibrium for each SNP was examined; rs16479 and rs16139 were excluded from the study because they did not reach equilibrium in the normal-BMI group. For this reason, we can neither confirm nor refute the association of the widely studied rs16139 with obesity and/or its traits in our population. The allele frequencies of the SNPs examined in the current study were similar to those reported for Caucasians in the HapMap (data not shown).

2.5. Statistical analysis

All continuous variables were expressed as the mean ± SEM. Normality distribution was assessed by the Kolmogorov–Smirnov test. Insulin, homeostasis model assessment for insulin resistance (HOMA-IR), total cholesterol, MMP-9, and total PAI-1 were logarithmically transformed, as they did not follow normality. Homogeneity of variances was estimated using the Levene test. Mean comparisons between obese and normal-BMI children for continuous variables were compared by Student's *t*-test for unpaired samples. The genotypic relative risk was assessed by comparing the obese group with the normal-BMI group and calculating the odds ratio (OR) and the 95% confidence interval (CI) using logistic regression analysis under an additive model adjusted by age and sex. Linear or logistic regressions in the entire population were performed under an additive model to estimate the association

of each SNP with phenotypic parameters related to obesity and biomarkers of inflammation and CVD risk. Haploview software version 4.2 was used with the default setting to assess LD between SNPs. The statistical analyses were performed with PLINK (version 1.07; Cambridge, MA) and SPSS (version 15.0.1; Chicago, IL).

3. Results

3.1. Children characteristics

Table 1 shows the characteristics of the obese and normal-BMI subjects. The anthropometric measurements (height, weight, BMI, BMI *z*-score, and waist circumference) as well as most of the clinical and metabolic markers (systolic and diastolic BP, insulin, HOMA-IR, and TAG) were significantly higher in the obese group than in the normal-BMI children. In contrast, total cholesterol and HDL-c levels were lower in the obese subjects, and glucose and LDL-c levels did not differ between the groups. The hepatic enzyme levels of alanine transaminase and γ-glutamyl transpeptidase were higher in the obese group, but the level of aspartate transaminase were lower. Adiponectin was also found at lower amounts in the obese group. Leptin, resistin, and inflammatory (PCR, IL-6, IL-8, and TNF-α) and CVD risk biomarkers (MPO, sE-selectin, sICAM-1, and PAI-1) were higher in the obese group than in the normal-BMI group, with the exception of MMP-9 levels, which did not significantly differ between the two groups.

Table 1
Anthropometric and biochemical parameters of the studied children.

	Normal-BMI	Obese	*P*
N	242	292	
Anthropometric characteristics			
Sex (M/F)	135/107	149/143	0.273
Age (y)	9.73 ± 0.2	9.43 ± 0.2	0.172
Weight (kg)	32.9 ± 0.7	55.9 ± 1.0	**<0.001**
Height (m)	1.37 ± 0.01	1.41 ± 0.01	**0.001**
BMI (kg/m^2)	17.14 ± 0.13	27.56 ± 0.24	**<0.001**
BMI *z*-score	−0.17 ± 0.04	3.49 ± 0.08	**<0.001**
Waist circumference (cm)	60.2 ± 0.5	84.0 ± 0.9	**<0.001**
Clinical and metabolic biomarkers			
Systolic BP (mm Hg)	97 ± 1	111 ± 1	**<0.001**
Diastolic BP (mm Hg)	60 ± 1	69 ± 1	**<0.001**
Glucose (mmol/L)	4.66 ± 0.02	4.72 ± 0.06	0.772
Insulin (pmol/L)	41.32 ± 1.60	79.59 ± 3.54	**<0.001**
HOMA-IR	1.26 ± 0.05	2.43 ± 0.12	**<0.001**
Triacylglycerols (mmol/L)	0.62 ± 0.01	0.85 ± 0.02	**<0.001**
Cholesterol (mmol/L)	4.43 ± 0.05	4.27 ± 0.05	**0.012**
HDL-c (mmol/L)	1.66 ± 0.03	1.32 ± 0.03	**<0.001**
LDL-c (mmol/L)	2.43 ± 0.05	2.51 ± 0.03	0.144
ALT (μkat/L)	0.28 ± 0.00	0.35 ± 0.00	**<0.001**
AST (μka/L)	0.40 ± 0.00	0.35 ± 0.00	**<0.001**
GGT (μka/L)	0.14 ± 0.00	0.18 ± 0.00	**<0.001**
Adipocytokines			
Adiponectin (mg/L)	28.28 ± 0.77	22.57 ± 0.66	**<0.001**
Resistin (μg/L)	9.65 ± 0.33	11.71 ± 0.34	**<0.001**
Leptin (μg/L)	4.30 ± 0.26	22.91 ± 0.86	**<0.001**
Inflammatory biomarkers			
C-reactive protein (nmol/L)	4.57 ± 0.67	18.95 ± 1.52	**<0.001**
IL-6 (ng/L)	4.50 ± 0.53	6.95 ± 0.75	**0.012**
IL-8 (ng/L)	1.57 ± 0.11	2.16 ± 0.15	**0.003**
TNF-α (ng/L)	3.06 ± 0.11	4.01 ± 0.13	**<0.001**
Cardiovascular disease risk biomarkers			
MMP-9 (μg/L)	79.99 ± 3.15	88.42 ± 3.90	0.636
MPO (μg/L)	13.10 ± 1.16	21.51 ± 1.70	**<0.001**
sE-Selectin (μg/L)	22.93 ± 0.76	31.26 ± 1.05	**<0.001**
sICAM-1 (mg/L)	0.153 ± 0.004	0.175 ± 0.005	**<0.001**
Active PAI-1 (μg/L)	5.16 ± 0.27	11.96 ± 0.57	**<0.001**
Total PAI-1 (μg/L)	18.95 ± 0.85	27.23 ± 1.01	**<0.001**

M: male; F: female; BMI: body mass index; BP: blood pressure; HOMA-IR: homeostasis model assessment for insulin resistance; HDL-c: high-density lipoprotein cholesterol; IL: interleukin; ALT: alanine transaminase; AST: aspartate transaminase; GGT: gamma-glutamyl transpeptidase; TNF-α: tumor necrosis factor alpha; MMP-9: metalloproteinase-9; MPO: myeloperoxidase; sICAM-1: soluble intracellular adhesion molecule-1, PAI-1: plasminogen activator inhibitor-1. The *P*-values < 0.05 are shown in bold.

3.2. Association of NPY gene SNPs with obesity

The association between the four analyzed *NPY* SNPs and obesity in children is shown in Table 2. Three out of the four SNPs were nominally associated with obesity after age and sex adjustment under an additive model. However, only the rs16131 and rs16147 SNPs remained statistically significant after Bonferroni correction (OR = 1.69 for allelic effect, 95% CI: 1.18–2.41, *P* = 0.004, Bonferroni corrected *P* = 0.016 and OR = 1.38 for allelic effect, 95% CI: 1.08–1.78, *P* = 0.010, Bonferroni corrected *P* = 0.040, respectively). No LD was observed between rs16147 located in the promoter region and the other studied intronic SNPs (rs16131 r^2 = 0.173, rs16178 r^2 = 0.409, and rs16135 r^2 = 0.002) in our population. Additionally, we found the LD in Caucasian Population for the *NPY* SNPs in the HapMap data, and we found that the functional Leu7Pro variant, rs16139, was not in LD with rs16131, rs16178 or rs16135. No data were available for rs16147.

3.3. Association of rs16147 with obesity-related traits

The rs16147 variant was associated with increased weight, BMI, and BMI *z*-score, as shown in Table 3. This SNP was also associated with lower levels of HDL-c; children carrying the T allele had a decrease of 0.06 mmol/L (95% CI: −0.10, −0.01) of HDL-c per allele. After an additional adjustment by BMI, the association with HDL-c was lost. No significant association of this SNP was observed with the other biomarkers studied.

3.4. Association of rs16131 with obesity-related traits

The rs16131 polymorphism was associated with increased height, weight, BMI, and BMI *z*-score (Table 4). It was also associated with higher insulin, HOMA-IR, TAG, and, leptin and lower HDL-c. Children carrying the risk allele had increases of 0.42 pmol/L (95% CI: 0.07, 0.69) of insulin, 0.06 (95% CI: 0.01, 0.10) of HOMA-IR, 0.08 mmol/L (95% CI: 0.02, 0.14) of TAG, and 3.29 μg/L (95% CI:

Table 2
Genotypic distribution of the *NPY* analyzed polymorphism and its association with obesity in children.

Polymorphism	Position	Allele 1/allele 2	Case			Control			Risk allele			OR (95%IC)	*P*	*P corr.*[*]
			11	12	22	11	12	22	Risk allele	Case	Control			
rs16147	Promoter	C/T	**69**	**128**	**82**	**70**	**123**	**43**	T	**0.526**	**0.444**	**1.38(1.08–1.78)**	**0.010**	**0.040**
rs16478	Intron 1	C/T	146	112	22	116	104	14	T	0.280	0.285	0.98(0.74–1.29)	0.867	1
rs16135	Intron 2	C/T	237	49	3	214	23	2	T	0.096	0.058	1.77(1.10–2.85)	0.019	0.078
rs16131	Intron 3	A/G	**207**	**66**	**17**	**192**	**48**	**1**	G	**0.171**	**0.104**	**1.69(1.18–2.41)**	**0.004**	**0.016**

Abbreviations: CI, confidence interval; OR, odds ration; SNP, single-nucleotide polymorphism. The OR for each SNP was adjusted by age and gender using an additive model.
[*] *P* values after Bonferroni correction.
The *P*-values < 0.05 are shown in bold.

Table 3
Association of the rs16147 SNP in the *NPY* gene with obesity related traits and with biomarkers of inflammation and cardiovascular disease risk in children.

	CC	TC	TT	β (95%IC)	*P*	P^a
N	139	251	125			
Anthropometric characteristics						
Height (m)	1.39 ± 0.01	1.39 ± 0.01	1.39 ± 0.01	−0.003(−0.01, 0.00)	0.121	0.548
Weight (kg)	45.0 ± 1.6	44.3 ± 1.2	48.1 ± 1.6	2.21(0.43, 4.00)	**0.016**	0.598
BMI	22.37 ± 0.52	22.36 ± 0.39	24.22 ± 0.53	1.03(0.30, 1.76)	**0.006**	–
BMI *z*-score	1.57 ± 0.18	1.68 ± 0.13	2.39 ± 0.20	0.38(0.12, 0.64)	**0.004**	–
Waist circumference (cm)	71.95 ± 1.55	72.17 ± 1.07	76.08 ± 1.39	1.84(−0.90, 4.58)	0.189	0.860
Clinical and metabolic biomarkers						
Systolic BP (mmHg)	104 ± 2	105 ± 1	106 ± 1	1.55(−0.27, 3.37)	0.096	0.870
Diastolic BP (mmHg)	64 ± 1	65 ± 1	65 ± 1	0.42(−1.07, 1.91)	0.584	0.447
Glucose (mmol/L)	4.72 ± 0.06	4.66 ± 0.02	4.72 ± 0.06	0.01(−0.04, 0.06)	0.684	0.667
Insulin (pmol/L)	61.05 ± 4.58	64.03 ± 3.61	59.24 ± 2.99	0.14(−0.07, 0.42)	0.169	0.986
HOMA-IR	1.88 ± 0.16	1.96 ± 0.12	1.80 ± 0.09	0.02(−0.01, 0.06)	0.178	0.988
Triacylglycerol (mmol/L)	0.75 ± 0.03	0.75 ± 0.02	0.73 ± 0.02	−0.003(−0.05, 0.04)	0.897	0.245
Cholesterol (mmol/L)	4.33 ± 0.05	4.35 ± 0.05	4.30 ± 0.08	−0.00003(−0.0003, 0.0003)	0.691	0.999
HDL-c (mmol/L)	1.50 ± 0.03	1.48 ± 0.03	1.40 ± 0.03	−0.06(−0.10, −0.01)	**0.018**	0.233
LDL-c (mmol/L)	94 ± 0.05	95 ± 0.05	96 ± 0.05	0.04(−0.04, 0.12)	0.329	0.358
Adipocytokines						
Adiponectin (mg/L)	25.36 ± 0.93	24.71 ± 0.77	26.21 ± 1.11	0.36(−1.02, 1.80)	0.628	0.208
Resistin (μg/L)	10.68 ± 0.42	10.66 ± 0.37	11.16 ± 0.52	0.28(−0.40, 0.97)	0.417	0.782
Leptin (μg/L)	13.98 ± 1.46	13.82 ± 0.86	16.42 ± 1.11	1.26(−0.50, 3.02)	0.160	0.300
Inflammatory biomarkers						
C-reactive protein (nmol/L)	21.24 ± 3.71	21.14 ± 2.57	24.86 ± 3.14	1.52(−3.33, 6.38)	0.537	0.783
IL-6 (ng/L)	6.14 ± 1.00	5.78 ± 0.61	6.09 ± 1.22	−0.05(−1.43, 1.32)	0.941	0.750
IL-8 (ng/L)	1.90 ± 0.22	1.89 ± 0.14	1.88 ± 0.18	−0.05(−0.32, 0.23)	0.744	0.487
TNF-α (ng/L)	3.45 ± 0.16	3.54 ± 0.13	3.78 ± 0.21	0.12(−0.13, 0.37)	0.346	0.739
Cardiovascular disease risk biomarkers						
MMP-9 (μg/L)	82.07 ± 4.47	86.99 ± 3.95	80.74 ± 5.28	−0.01(−0.05, 0.02)	0.395	0.394
MPO (μg/L)	17.71 ± 2.06	18.17 ± 1.57	17.23 ± 2.43	−0.43(−3.52, 2.66)	0.785	0.435
sE-Selectin (μg/L)	26.46 ± 1.22	27.95 ± 0.96	26.58 ± 1.66	0.09(−1.83, 2.00)	0.931	0.569
sICAM-1 (mg/L)	0.165 ± 0.007	0.167 ± 0.004	0.162 ± 0.005	−0.003(−0.01, 0.01)	0.478	0.275
Active PAI-1 (μg/L)	8.15 ± 0.74	9.33 ± 0.56	8.92 ± 0.66	0.48(−0.55, 1.51)	0.357	0.743
Total PAI-1 (μg/L)	22.16 ± 1.20	25.05 ± 1.19	21.51 ± 1.34	−0.01(−0.05, 0.03)	0.653	0.228

β Coefficients represent the change in absolute traits values of each additional risk allele. General linear models were used to examine associations with the assumption of an additive effect. *P* adjusted by age and sex. P^a adjusted by age, sex and BMI. The *P*-values < 0.05 are shown in bold. CI: Confidence interval; BMI: body mass index; BP: blood pressure; HOMA-IR: homeostasis model assessment for insulin resistance; HDL-c: high-density lipoprotein cholesterol; IL: interleukin; TNF-α: tumor necrosis factor alpha; MMP-9: metalloproteinase-9; MPO: myeloperoxidase; sICAM-1: soluble intracellular adhesion molecule-1, PAI-1: plasminogen activator inhibitor.

0.88, 5.70) of leptin and an HDL-c decrease of 0.08 mmol/L (95% CI: −0.15, −0.02) per allele. After an additional adjustment by BMI, only TAG showed a trend of association with this SNP ($P = 0.072$). We performed a multiple regression analysis of the metabolic syndrome components and rs16131, and the plasma concentration of TAG was the only component independently associated with this polymorphism ($\beta = 1.007$, 95% CI: 1.002–1.013, $P = 0.012$).

4. Discussion

In the present study, we validate the association between the *NPY* rs16147 genotype and BMI in Spanish children, observing higher BMI values in TT homozygotes as compared with heterozygous C allele carriers. Moreover, we find for the first time a significant association of the intronic rs16131 SNP with higher BMI, insulin, HOMA-IR, TAG, and leptin and lower HDL-c.

The rs16147 variant, located in the promoter region of the gene, is shown in our study to be associated with obesity and lower levels of HDL-c. These findings correspond well with the recent work of Hohmann et al., who also found an association of this variant with BMI development from infancy to adulthood [7]. Hohmann et al. also provided evidence of a significant age dependence of this association, indicating that the NPY effect increased with age and was more pronounced in older age groups than in younger ones. Our results are in concordance with these findings, as they show association from eight years onwards, and our cohort of children is between 5 and 15 years old, with a mean of 9.6 years. More evidence of the influence of this SNP on obesity has been demonstrated in post-mortem brain and lymphoblastoid cell lines that show higher *NPY* expression in risk allele carriers (T) of rs16147 [33]. As NPY leads to an increase in food intake as well as the number of meals consumed per day and directly stimulates adipocyte growth in mice, the higher BMI scores of T allele carriers in the present study suggests there may be a corresponding increase in *NPY* expression levels.

Interestingly, in the present study, rs16131 was not only associated with the same features as rs16147 but also with higher levels of TAG, insulin, HOMA-IR, and leptin, most of which are features of metabolic syndrome. It has been suggested that NPY may be involved in the development of insulin resistance [24], and our results add further support to this hypothesis. In fact, our results show that plasma concentration of TAG was the only metabolic syndrome component independently associated with the rs16131 polymorphism. This finding suggests that the rs16131 variant of the *NPY* gene, rather than being involved in the regulation of energy intake, might be related to the action of this neuropeptide on the key organs involved in the synthesis and use of TAG, such as liver and adipose tissue. Van den Hoek et al. [27] demonstrated that the cerebroventricular administration of NPY in rats induces insulin resistance through the activation of the sympathetic nerve endings reaching the liver. Furthermore, Ruohonens et al. [20] demonstrated in transgenic DβH-NPY mice that an overexpression of *NPY* in the central nervous system and noradrenergic neurons of the brain causes an increase in adiposity and the hepatic accumulation of TAG, generating hyperinsulinemia and glucose metabolism dysfunction over time; these changes were independent of NPY concentrations in the hypothalamus. Additionally, it has been demonstrated that an intact hepatic sympathetic innervation and arcuate nucleus are also necessary to maintain VLDL (very low density lipoproteins)-TAG secretion during fasting,

Table 4
Association of the rs16131 SNP in the *NPY* gene with obesity related traits and with biomarkers of inflammation and cardiovascular disease risk biomarkers in children.

	AA	AG	GG	β (95%IC)	*P*	P^a
N	399	114	18			
Anthropometric factors						
Height (m)	1.39 ± 0.01	1.38 ± 0.01	1.40 ± 0.02	−0.01(−0.01, −0.001)	**0.034**	0.219
Weight (kg)	45.1 ± 0.9	45.2 ± 1.7	54.2 ± 3.0	2.97(0.55, 5.40)	**0.017**	0.551
BMI	22.59 ± 0.30	23.01 ± 0.60	27.36 ± 1.01	1.40(0.41, 2.39)	**0.006**	–
BMI *z*-score	1.73 ± 0.11	1.90 ± 0.20	3.59 ± 0.41	0.47(0.12, 0.82)	**0.008**	–
Waist circumference (cm)	72.64 ± 0.88	73.49 ± 1.46	81.50 ± 2.95	0.62(−3.13, 4.37)	0.747	0.800
Clinical and metabolic biomarkers						
Systolic BP (mmHg)	105 ± 1	105 ± 1	107 ± 4	0.71(−1.78, 3.20)	0.578	0.404
Diastolic BP (mmHg)	64 ± 1	65 ± 1	64 ± 3	0.62(−1.41, 2.64)	0.550	0.627
Glucose (mmol/L)	4.72 ± 0.02	4.66 ± 0.06	4.83 ± 0.11	0.01(−0.05, 0.08)	0.657	0.657
Insulin (pmol/L)	61.12 ± 2.50	62.02 ± 5.28	88.13 ± 9.58	0.42(0.07, 0.69)	**0.015**	0.225
HOMA-IR	1.88 ± 0.08	1.86 ± 0.16	2.73 ± 0.31	0.06(0.01, 0.10)	**0.018**	0.243
Triacylglycerol (mmol/L)	0.72 ± 0.01	0.81 ± 0.05	0.84 ± 0.07	0.08(0.02, 0.14)	**0.009**	0.072
Cholesterol (mmol/L)	4.35 ± 0.03	4.35 ± 0.08	4.17 ± 0.18	−0.0001(−0.001, 0.0003)	0.527	0.803
HDL-c (mmol/L)	1.50 ± 0.03	1.42 ± 0.03	1.30 ± 0.08	−0.08(−0.15, −0.02)	**0.009**	0.128
LDL-c (mmol/L)	2.46 ± 0.03	2.51 ± 0.08	2.46 ± 0.16	0.04(−20.07, 0.15)	0.480	0.521
Adipocytokines						
Adiponectin (mg/L)	25.18 ± 0.57	24.86 ± 1.21	25.81 ± 3.37	−0.25(−2.12, 1.70)	0.799	0.635
Resistin (μg/L)	10.60 ± 0.27	11.61 ± 0.64	9.68 ± 0.56	0.41(−0.53, 1.36)	0.392	0.758
Leptin (μg/L)	13.72 ± 0.74	15.99 ± 1.34	22.20 ± 2.28	3.29(0.88, 5.70)	**0.008**	0.412
Inflammatory biomarkers						
C-reactive protein (nmol/L)	21.91 ± 3.14	19.14 ± 2.29	33.24 ± 4.48	−0.19(−6.38, 6.76)	0.675	0.459
IL-6 (ng/L)	5.85 ± 0.55	6.67 ± 1.15	2.52 ± 0.45	−0.30(−2.17, 1.57)	0.750	0.554
IL-8 (ng/L)	1.85 ± 0.11	2.04 ± 0.22	1.81 ± 0.23	0.07(−0.31, 0.44)	0.730	0.982
TNF-α (ng/L)	3.47 ± 0.10	3.79 ± 0.18	4.16 ± 0.57	0.26(0.08, 0.59)	0.139	0.383
Cardiovascular disease risk biomarkers						
MMP-9 (μg/L)	82.87 ± 2.94	88.45 ± 5.50	84.72 ± 16.46	−0.002(−0.05, 0.04)	0.927	0.913
MPO (μg/L)	17.42 ± 1.21	19.28 ± 2.74	12.74 ± 2.46	−0.28(−4.44, 3.88)	0.895	0.514
sE-Selectin (μg/L)	27.58 ± 0.81	26.35 ± 1.35	26.32 ± 4.01	−1.30(−3.98, 1.39)	0.345	0.050
sICAM-1 (mg/L)	0.163 ± 0.004	0.168 ± 0.006	0.171 ± 0.014	0.002(−0.01, 0.01)	0.681	0.955
Active PAI-1 (μg/L)	8.60 ± 0.43	9.33 ± 0.79	11.70 ± 1.63	1.01(−0.38, 2.41)	0.155	0.819
Total PAI-1 (μg/L)	23.11 ± 0.81	23.07 ± 1.72	32.15 ± 5.86	0.02(−0.04, 0.07)	0.549	0.910

β Coefficients represent the change in absolute traits values of each additional risk allele. General linear models were used to examine associations with the assumption of an additive effect. *P* adjusted by age and sex. P^a adjusted by age, sex and BMI. The *P*-values < 0.05 are shown in bold. CI: Confidence interval; BMI: body mass index; BP: blood pressure; HOMA-IR: homeostasis model assessment for insulin resistance; HDL-c: high-density lipoprotein cholesterol; IL: interleukin; TNF-α: tumor necrosis factor alpha; MMP-9: metalloproteinase-9; MPO: myeloperoxidase; sICAM-1: soluble intracellular adhesion molecule-1, PAI-1: plasminogen activator inhibitor-1.

and the increased release of hypothalamic NPY maintains this lipoprotein secretion via the sympathetic input to the liver. This mechanism is important during fasting; however, it could play a pathophysiological role in conditions characterized by a constantly high level of NPY activity, as is found in animal models of obesity and hypertriglyceridemia [4]. Moreover, a recent study by Rojas et al. [19] demonstrated that the effects of NPY on feeding and/or weight gain are relatively dissociable from the effects on hepatic VLDL-TAG secretion. Although functional studies of the rs16131 variant are necessary, it is possible that this SNP, located in the third intron of the gene, tagged a causal novel variant that interacts with NPY function, potentially leading to an increase in plasma TAG and an early onset of metabolic syndrome. It has been demonstrated that high sympathetic activity and low parasympathetic activity significantly correlate with components of metabolic syndrome, including hypertriglyceridemia [14]. However, further studies in larger populations will be necessary to validate this association.

Our study has several strengths and limitations to highlight. Among the strengths of this study are the high quantity of biomarkers measured in the children and the method of SNP selection; we selected SNPs from a candidate gene to design a case–control association study as a complementary strategy to the genome-wide association studies for the identification of gene variants associated with obesity and its comorbidities. One limitation is that the sample size of the current study is relatively small for a genetic association study. Because these types of studies are capable of producing false positive results, the obesity association of rs16131 reported here must currently be viewed as a preliminary result, which requires further validation in a larger number of independent samples. Another limitation of this study is that a number of environmental factors known to influence body weight in children were not taken into account. Furthermore, information on our cohort's nutrition and level of physical activity, which are factors strongly associated with body-weight regulation, was not available.

5. Conclusion

We confirm the association of rs16147 with obesity in a cohort of Spanish children. Our results suggest that the rs16131 risk allele influences the early onset of obesity and metabolic syndrome features, particularly the elevation of plasma TAG. The question of how these polymorphisms actively play a role in obesity and metabolic syndrome remains unanswered.

Competing interests

The authors declare that they have no competing interests.

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References

[1] Billington CJ, Briggs JE, Grace M, Levine AS. Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. Am J Physiol 1991;260:R321–7.
[2] Bray MS, Boerwinkle E, Hanis CL. Sequence variation within the neuropeptide Y gene and obesity in Mexican Americans. Obes Res 2000;8:219–26.
[3] Brothers SP, Wahlestedt C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. EMBO Mol Med 2010;2:429–39.
[4] Bruinstroop E, Pei L, Ackermans MT, Foppen E, Foppen E, Borgers AJ, et al. Hypothalamic neuropeptide Y (NPY) controls hepatic VLDL-triglyceride secretion in rats via the sympathetic nervous system. Diabetes 2012;61:1043–50.
[5] Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000;320:1240–3.
[6] Ding B, Kull B, Liu Z, Mottagui-Tabar S, Thonberg H, Gu HF, et al. Human neuropeptide Y signal peptide gain-of-function polymorphism is associated with increased body mass index: possible mode of function. Regul Pept 2005;127:45–53.
[7] Hohmann S, Buchmann AF, Witt SH, Rietschel M, Jennen-Steinmetz C, Schmidt MH, et al. Increasing association between a neuropeptide Y promoter polymorphism and body mass index during the course of development. Pediatr Obes 2012;7:453–60.
[8] Jaakkola U, Kakko T, Juonala M, Lehtimäki T, Viikari J, Jääskeläinen AE, et al. Neuropeptide Y polymorphism increases the risk for asthma in overweight subjects; protection from atherosclerosis in asthmatic subjects – the cardiovascular risk in young Finns study. Neuropeptides 2012;46:321–8.
[9] Karvonen MK, Koulu M, Pesonen U, Uusitupa MI, Tammi A, Viikari J, et al. Leucine 7 to proline 7 polymorphism in the preproneuropeptide Y is associated with birth weight and serum triglyceride concentration in preschool aged children. J Clin Endocrinol Metab 2000;85:1455–60.
[10] Karvonen MK, Pesonen U, Koulu M, Niskanen L, Laakso M, Rissanen A, et al. Association of a leucine (7)-to-proline (7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. Nat Med 1998;4:1434–7.
[11] Karvonen MK, Valkonen VP, Lakka TA, Salonen R, Koulu M, Pesonen U, et al. Leucine7 to proline7 polymorphism in the preproneuropeptide Y is associated with the progression of carotid atherosclerosis, blood pressure and serum lipids in Finnish men. Atherosclerosis 2001;159:145–51.
[12] Kim NS, Oh SM, Ko MM, Cha MH, Kang BK, Bang OS. Association of the C-399T promoter polymorphism of neuropeptide Y with susceptibility to ischemic stroke. Clin Biochem 2009;42:1699–704.
[13] Lee C, Kong M. An interactive association of common sequence variants in the neuropeptide Y gene with susceptibility to ischemic stroke. Stroke 2007;38:2663–9.
[14] Licht CM, Vreeburg SA, van Reedt Dortland AK, Giltay EJ, Hoogendijk WJ, DeRijk RH, et al. Increased sympathetic and decreased parasympathetic activity rather than changes in hypothalamic–pituitary–adrenal axis activity is associated with metabolic abnormalities. J Clin Endocrinol Metab 2010;95:2458–66.
[15] Masoudi Kazemabad A, Jamialahmadi K, Moohebati M, Mojarrad M, Manshadi RD, Akhlaghi S, et al. Neuropeptide Y Leu7Pro polymorphism associated with the metabolic syndrome and its features in patients with coronary artery disease. Angiology 2013;64:40–5.
[16] McCrindle BW. Assessment and management of hypertension in children and adolescent. Nat Rev Cardiol 2010;7:155–63.
[17] Niskanen L, Karvonen MK, Valve R, Koulu M, Pesonen U, Mercuri M, et al. Leucine 7 to proline 7 polymorphism in the neuropeptide Y gene is associated with enhanced carotid atherosclerosis in elderly patients with type 2 diabetes and control subjects. J Clin Endocrinol Metab 2000;85:2266–9.
[18] Nordman S, Ding B, Ostenson CG, Kärvestedt L, Brismar K, Efendic S, et al. Leu7Pro polymorphism in the neuropeptide Y (NPY) gene is associated with impaired glucose tolerance and type 2 diabetes in Swedish men. Exp Clin Endocrinol Diabetes 2005;113:282–7.
[19] Rojas JM, Stafford JM, Saadat S, Printz RL, Beck-Sickinger AG, Niswender KD. Central nervous system neuropeptide Y signaling via the Y1 receptor partially dissociates feeding behavior from lipoprotein metabolism in lean rats. Am J Physiol Endocrinol Metab 2012;303:E1479–88.
[20] Ruohonen ST, Pesonen U, Moritz N, Kaipio K, Roytta M, Koulu M, et al. Transgenic mice overexpressing neuropeptide Y in noradrenergic neurons. Diabetes 2008;57:1517–25.
[21] Sainsbury A, Rohner-Jeanrenaud F, Cusin I, Zakrzewska KE, Halban PA, Gaillard RC, et al. Chronic central neuropeptide Y infusion in normal rats: status of the hypothalamo–pituitary–adrenal axis, and vagal mediation of hyperinsulinaemia. Diabetologia 1997;40:1269–77.
[22] Salminen M, Lehtimaki T, Fan YM, Vahlberg T, Kivela SL. Leucine 7 to proline 7 polymorphism in the neuropeptide Y gene and changes in serum lipids during a family-based counselling intervention among school-aged children with a family history of CVD. Public Health Nutr 2008;11:1156–62.
[23] Shah SH, Freedman NJ, Zhang L, Crosslin DR, Stone DH, Haynes C, et al. Neuropeptide Y gene polymorphisms confer risk of early-onset atherosclerosis. PLoS Genet 2009;5:e1000318.
[24] Singhal NS, Lazar MA, Ahima RS. Central resistin induces hepatic insulin resistance via neuropeptide Y. J Neurosci 2007;27:12924–32.
[25] Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides 1986;7:1189–92.
[26] Ukkola O, Kesaniemi YA. Leu7Pro polymorphism of PreproNPY associated with an increased risk for type II diabetes in middle-aged subjects. Eur J Clin Nutr 2007;61:1102–5.
[27] van den Hoek AM, van Heijningen C, Schröder-van der Elst JP, Ouwens DM, Havekes LM, Romijn JA, et al. Intracerebroventricular administration of neuropeptide Y induces hepatic insulin resistance via sympathetic innervation. Diabetes 2008;57:2304–10.
[28] van Rossum CT, Pijl H, Adan RA, Hoebee B, Seidell JC. Polymorphisms in the NPY and AGRP genes and body fatness in Dutch adults. Int J Obes (Lond) 2006;30:1522–8.
[29] Vettor R, Zarjevski N, Cusin I, Rohner-Jeanrenaud F, Jeanrenaud B. Induction and reversibility of an obesity syndrome by intracerebroventricular neuropeptide Y administration to normal rats. Diabetologia 1994;37:1202–8.
[30] Yeung EH, Zhang C, Chen J, Bowers K, Hu FB, Kang G, et al. Polymorphisms in the neuropeptide Y gene and the risk of obesity: findings from two prospective cohorts. J Clin Endocrinol Metab 2011;96:E2055–62.
[31] Yu JT, Yu NN, Gao SS, Song JH, Ma T, Wang ND, et al. Neuropeptide Y polymorphisms and ischemic stroke in Chinese population. Clin Chim Acta 2010;411:242–5.
[32] Zhang X, Qi Q, Liang J, Hu FB, Sacks FM, Qi L. Neuropeptide y promoter polymorphism modifies effects of a weight-loss diet on 2-year changes of blood pressure: the preventing overweight using novel dietary strategies trial. Hypertension 2012;60:1169–75.
[33] Zhou Z, Zhu G, Hariri AR, Enoch MA, Scott D, Sinha R, et al. Genetic variation in human NPY expression affects stress response and emotion. Nature 2008;452:997–1001.

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs7688274	*ALB*	4	74487522	G	0.383	0.401	5'-UTR	0.909	0.534	
rs3756066	*ALB*	4	74488552	A	0.408	0.402	5'-UTR	0.921	0.587	
rs3775485	*ALB*	4	74494685	T	0.417	0.422	tag SNP	0.963	0.803	
rs11538228	*ALB*	4	74494930	G	0.000	0.000	missense			MAF
rs7678298	*ALB*	4	74503292	A	0.165	0.141	tag SNP	0.833	0.385	
rs17593621	*AOX1*	2	201157141	G	0.208	0.275	5'-UTR	0.811	0.224	
rs12621063	*AOX1*	2	201157687	A	0.142	0.172	5'-UTR	1.288	0.205	
rs2072034	*AOX1*	2	201157979	G	0.150	0.176	5'-UTR	1.306	0.180	
rs16833793	*AOX1*	2	201158132	A	0.136	0.136	5'-UTR	0.949	0.810	
rs1527944	*AOX1*	2	201164490	A	0.248	0.217	tag SNP	1.029	0.877	
rs2463489	*AOX1*	2	201168561	A	0.150	0.176	tag SNP	1.307	0.178	
rs4674211	*AOX1*	2	201169086	C	0.102	0.136	tag SNP	0.949	0.810	
rs6742109	*AOX1*	2	201174977	A	0.438	0.416	tag SNP	0.954	0.756	
rs2293528	*AOX1*	2	201177394	G	0.332		tag SNP			CALL
rs35128788	*AOX1*	2	201182080	C	ND	0.000	missense			MAF
rs6753383	*AOX1*	2	201192310	A	0.319	0.342	tag SNP	0.812	0.201	
rs35217482	*AOX1*	2	201207801	T	ND	0.000	missense			MAF
rs2256977	*AOX1*	2	201208428	A	0.469	0.449	tag SNP	0.896	0.482	
rs17533238	*AOX1*	2	201209676	G	0.158	0.156	tag SNP	1.320	0.198	
rs41309768	*AOX1*	2	201209936	T	ND	0.000	missense			MAF
rs2470900	*AOX1*	2	201211143	A	0.075	0.084	tag SNP	1.068	0.806	
rs2465663	*AOX1*	2	201211421	G	0.279		tag SNP			CALL
rs2540069	*AOX1*	2	201228843	A	0.341	0.299	tag SNP	1.268	0.156	
rs3815502	*AOX1*	2	201234165	C	0.230	0.151	tag SNP	1.196	0.448	
rs35345784	*AOX1*	2	201235894	T	ND	0.000	missense			MAF
rs3731722	*AOX1*	2	201242634	G	0.033	0.036	missense	0.415	0.047	
rs2540066	*AOX1*	2	201243320	G	0.473	0.441	tag SNP	1.174	0.294	
rs1050887	*AOX1*	2	201244175	A	0.050	0.036	3'-UTR	0.415	0.047	
rs2715906	*AOX1*	2	201244708	A	0.492	0.401	3'-UTR	1.305	0.082	
rs564250	*CAT*	11	34415437	A	0.169	0.190	5'-UTR	0.997	0.987	
rs769214	*CAT*	11	34416293	G	0.299	0.346	5'-UTR	1.418	0.035	
rs1001179	*CAT*	11	34416807	A	0.248		5'-UTR			CALL
rs7943316	*CAT*	11	34417048	T	0.275	0.349	5'-UTR	1.382	0.050	
rs1049982	*CAT*	11	34417117	A	0.292	0.347	5'-UTR	1.408	0.039	
rs525938	*CAT*	11	34420169	G	0.301	0.325	tag SNP	1.289	0.127	
rs494024	*CAT*	11	34421324	A	0.412	0.345	tag SNP	0.734	0.051	
rs769218	*CAT*	11	34427255	A	0.195	0.272	tag SNP	1.302	0.126	
rs12269988	*CAT*	11	34429350	G	0.058	0.035	tag SNP	0.774	0.542	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs2073058	*CAT*	11	34434649	G	0.230	0.289	tag SNP	1.114	0.529	
rs4755374	*CAT*	11	34443180	C	0.124	0.131	tag SNP	1.031	0.892	
rs12803540	*CAT*	11	34449323	G	0.071	0.141	tag SNP	1.354	0.160	
rs7104301	*CAT*	11	34450214	G	0.233	0.291	3'-UTR	1.133	0.467	
rs475043	*CAT*	11	34450377	G	0.458	0.342	3'-UTR	0.714	0.035	
rs2280789	*CCL5*	17	31231116	G	0.102	0.141	tag SNP	0.710	0.109	
rs2107538	*CCL5*	17	31231893	A	0.095	0.175	3'-UTR	0.793	0.221	
rs28912068	*CCL5*	17	31233084	T	0.143		3'-UTR			CALL
rs503250	*CCS*	11	66128642	G	0.438	0.461	tag SNP	1.019	0.902	
rs1205	*CRP*	1	157948857	A	0.341	0.323	tag SNP	1.115	0.507	
rs1130864	*CRP*	1	157949715	A	0.309	0.338	tag SNP	0.904	0.529	
rs34340208	*CRP*	1	157950117	A	ND	0.000	missense			MAF
rs34200896	*CRP*	1	157950216	T	ND	0.000	missense			MAF
rs34672691	*CRP*	1	157950415	C	ND	0.000	missense			MAF
rs3091244	*CRP*	1	157951289	A	0.048	0.365	3'-UTR	0.894	0.455	
rs2794521	*CRP*	1	157951720	C	0.348	0.000	3'-UTR			MAF
rs7195830	*CYBA*	16	87237213	A	0.402	0.366	tag SNP	0.833	0.248	
rs1049254	*CYBA*	16	87237329	A	0.517	0.371	missense	0.812	0.188	
rs4673	*CYBA*	16	87240737	A	0.342	0.389	missense	1.053	0.751	
rs11547387	*CYBA*	16	87241034	C	ND	0.022	missense	1.036	0.945	
rs3794624	*CYBA*	16	87244575	A	0.398	0.303	tag SNP	0.865	0.408	
rs8854	*CYBA*	16	87246366	A	0.079	0.041	3'-UTR	0.736	0.407	
rs8189	*CYGB*	17	72035386	A	0.367		tag SNP			CALL
rs4648336	*CYGB*	17	72038233	A	0.128	0.123	tag SNP	1.011	0.962	
rs9902524	*CYGB*	17	72041153	A	0.175		tag SNP			CALL
rs4647879	*CYGB*	17	72042640	A	0.283	0.218	tag SNP	1.038	0.839	
rs2280209	*DGKG*	3	187347934	C	0.358	0.341	3'-UTR	1.196	0.239	
rs9835936	*DGKG*	3	187348937	A	0.475	0.483	3'-UTR	0.928	0.603	
rs13089860	*DGKG*	3	187349309	A	0.362	0.337	3'-UTR	1.209	0.211	
rs6444109	*DGKG*	3	187349977	A	0.327	0.337	3'-UTR	1.209	0.211	
rs2575	*DGKG*	3	187350085	A	0.475	0.483	3'-UTR	0.928	0.603	
rs2574	*DGKG*	3	187350244	G	0.483	0.499	3'-UTR	1.007	0.964	
rs3732940	*DGKG*	3	187350331	A	0.150	0.158	3'-UTR	0.781	0.208	
rs34284245	*DGKG*	3	187388761	A	ND	0.000	missense			MAF
rs3213770	*DGKG*	3	187469292	T	0.000	0.000	missense			MAF
rs2193587	*DGKG*	3	187472790	G	0.142	0.232	missense	0.984	0.926	
rs1004588	*DGKG*	3	187489312	G	0.480		missense			CALL
rs35545745	*DGKG*	3	187498552	A	0.000	0.000	missense			MAF

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs7617588	*DGKG*	3	187562684	G	0.133	0.176	5'-UTR	0.711	0.086	
rs669181	*DHCR24*	1	55087676	A	0.308	0.330	5'-UTR	0.900	0.502	
rs7373	*DHCR24*	1	55088199	C	0.308	0.332	tag SNP	0.923	0.609	
rs7374	*DHCR24*	1	55088910	G	0.302	0.332	tag SNP	0.918	0.584	
rs1138030	*DHCR24*	1	55090273	A	0.181	0.240	tag SNP	0.930	0.660	
rs12025718	*DHCR24*	1	55100528	G	0.210	0.312	tag SNP	0.825	0.217	
rs608458	*DHCR24*	1	55109538	C	0.270	0.404	tag SNP	0.793	0.121	
rs601902	*DHCR24*	1	55122611	A	0.119	0.085	tag SNP	1.148	0.601	
rs2274941	*DHCR24*	1	55125958	C	0.417	0.367	3'-UTR	0.978	0.883	
rs6588537	*DHCR24*	1	55126781	A	0.112	0.084	3'-UTR	1.122	0.669	
rs4927176	*DHCR24*	1	55126923	G	0.408	0.486	3'-UTR	0.802	0.130	
rs1365242	*DUOX1*	15	43208010	C	0.458	0.478	5'-UTR	1.000	0.998	
rs1648281	*DUOX1*	15	43209050	G	0.242	0.224	5'-UTR	1.013	0.943	
rs17595239	*DUOX1*	15	43211181	A	0.229	0.196	tag SNP	1.013	0.942	
rs2292464	*DUOX1*	15	43221785	A	0.438	0.497	tag SNP	0.987	0.928	
rs16939743	*DUOX1*	15	43230188	G	0.000	0.007	missense	1.616	0.603	
rs16939752	*DUOX1*	15	43231425	G	0.117	0.141	missense	1.170	0.464	
rs2458236	*DUOX1*	15	43233448	G	0.258	0.356	missense	0.770	0.105	
rs2292466	*DUOX1*	15	43235364	A	0.000	0.000	missense			MAF
rs7169193	*DUOX1*	15	43244419	T	0.438		tag SNP			CALL
rs1648312	*DUOX1*	15	43244641	G	0.375	0.489	tag SNP	0.864	0.322	
rs2292467	*DUOX1*	15	43244750	A	0.258	0.354	3'-UTR	0.765	0.100	
rs1648311	*DUOX1*	15	43244811	A	0.290		3'-UTR			CALL
rs269868	*DUOX2*	15	43179367	G	0.017	0.081	missense	0.703	0.212	
rs2277611	*DUOX2*	15	43179698	T	0.000	0.000	missense			MAF
rs269866	*DUOX2*	15	43181698	G	0.389	0.370	tag SNP	0.865	0.338	
rs8028305	*DUOX2*	15	43185715	A	ND	0.000	missense			MAF
rs2467827	*DUOX2*	15	43190991	T	ND		missense			CALL
rs2001616	*DUOX2*	15	43191358	G	0.036	0.086	missense	0.659	0.140	
rs1050450	*GPX1*	3	49369838	A	0.194	0.254	3'-UTR	1.294	0.227	HW
rs8179169	*GPX1*	3	49370702	G	0.022	0.000	missense			MAF
rs3811699	*GPX1*	3	49371364	G	0.194	0.315	3'-UTR	1.211	0.212	
rs3448	*GPX1*	3	49371755	A	0.300	0.227	3'-UTR	0.936	0.716	
rs12172810	*GPX2*	14	64475135	A	0.177	0.191	5'-UTR	1.010	0.960	
rs11623705	*GPX2*	14	64475475	A	0.142	0.084	5'-UTR	1.099	0.730	
rs17880492	*GPX2*	14	64476096	T	0.004		tag SNP			CALL
rs2412065	*GPX2*	14	64477410	G	0.235	0.190	tag SNP	1.018	0.928	
rs2737844	*GPX2*	14	64478262	A	0.310	0.324	tag SNP	0.940	0.695	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs1800669	*GPX2*	14	64478957	T	0.050	0.027	tag SNP	1.655	0.285	
rs17102360	*GPX2*	14	64480073	G	0.050	0.046	3'-UTR	1.674	0.170	
rs2296327	*GPX2*	14	64480300	A	0.225	0.170	3'-UTR	1.181	0.387	
rs4902347	*GPX2*	14	64480953	A	0.150	0.078	3'-UTR	1.192	0.542	
rs2042236	*GPX3*	5	150378308	A	0.058	0.120	5'-UTR	1.479	0.085	
rs1946234	*GPX3*	5	150379403	C	0.058	0.123	5'-UTR	1.312	0.219	
rs1946236	*GPX3*	5	150379484	T	0.058	0.123	5'-UTR	1.312	0.219	
rs8177406	*GPX3*	5	150379827	G	0.067	0.123	5'-UTR	1.312	0.219	
rs8177409	*GPX3*	5	150380043	T	0.059	0.123	5'-UTR	1.312	0.219	
rs8177412	*GPX3*	5	150380280	G	0.129	0.120	tag SNP	1.431	0.119	
rs4958872	*GPX3*	5	150382527	G	0.283	0.293	tag SNP	0.993	0.967	
rs8177427	*GPX3*	5	150383204	A	0.217	0.193	tag SNP	0.824	0.307	
rs8177431	*GPX3*	5	150384189	G	0.358	0.358	tag SNP	1.119	0.461	
rs8177445	*GPX3*	5	150387208	C	0.000	0.000	missense			MAF
rs11548	*GPX3*	5	150387976	A	0.050	0.060	tag SNP	1.454	0.257	
rs2070593	*GPX3*	5	150388133	A	0.108	0.157	tag SNP	1.364	0.130	
rs4661	*GPX3*	5	150388508	T	0.050	0.000	3'-UTR			MAF
rs757228	*GPX4*	19	1052992	G	0.475	0.497	5'-UTR	0.729	0.048	
rs757229	*GPX4*	19	1053114	C	0.468		5'-UTR			CALL
rs3746165	*GPX4*	19	1053211	G	0.475	0.499	5'-UTR	0.736	0.055	
rs8103188	*GPX4*	19	1054465	G	0.433	0.444	5'-UTR	0.696	0.026	
rs8178967	*GPX4*	19	1055047	A	0.000	0.000	missense			MAF
rs4851	*GPX4*	19	1057635	A	ND	0.194	missense	1.225	0.352	HW
rs2075710	*GPX4*	19	1057845	A	0.194	0.250	3'-UTR	0.933	0.699	
rs2074451	*GPX4*	19	1058035	A	0.500	0.465	3'-UTR	1.143	0.401	
rs2074452	*GPX4*	19	1058160	A	0.258	0.277	3'-UTR	0.948	0.761	
rs377514	*GPX5*	6	28599929	G	0.093	0.129	5'-UTR	0.965	0.878	
rs28382586	*GPX5*	6	28600461	C	0.136	0.147	5'-UTR	1.544	0.051	
rs28382587	*GPX5*	6	28600558	A	0.091		5'-UTR			CALL
rs28745594	*GPX5*	6	28600624	A	0.068		5'-UTR			CALL
rs28382590	*GPX5*	6	28601564	G	0.088		5'-UTR			CALL
rs445870	*GPX5*	6	28602306	G	0.354	0.330	tag SNP	1.452	0.027	
rs769188	*GPX5*	6	28607545	G	0.000	0.000	tag SNP			MAF
rs380879	*GPX5*	6	28608428	C	0.080	0.124	tag SNP	1.046	0.850	
rs451774	*GPX5*	6	28610529	G	0.314		3'-UTR			CALL
rs35604709	*GPX6*	6	28579276	A	0.091	0.078	3'-UTR	1.021	0.944	
rs9468385	*GPX6*	6	28579591	A	0.159	0.126	tag SNP	0.966	0.883	
rs35701070	*GPX6*	6	28580151	C	0.000	0.000	missense			MAF

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs34955392	*GPX6*	6	28580232	G	0.000		missense			CALL
rs35394555	*GPX6*	6	28581510	G	0.024	0.008	missense	0.410	0.314	
rs35062161	*GPX6*	6	28586534	A	0.068		missense			CALL
rs406113	*GPX6*	6	28591461	C	0.285	0.352	missense	1.562	0.009	
rs6921021	*GPX6*	6	28591569	G	0.167	0.137	3'-UTR	1.084	0.723	
rs11757000	*GPX6*	6	28592848	G	0.175	0.147	3'-UTR	1.544	0.051	
rs1416093	*GPX7*	1	52839622	A	0.182	0.264	5'-UTR	0.946	0.749	
rs6588431	*GPX7*	1	52840174	A	0.367	0.388	5'-UTR	0.985	0.924	
rs11810754	*GPX7*	1	52840502	A	0.294	0.394	5'-UTR	1.005	0.973	
rs1047635	*GPX7*	1	52847120	C	0.433	0.443	tag SNP	0.979	0.889	
rs4451503	*GPX7*	1	52847568	C	0.238	0.000	3'-UTR			MAF
rs2020916	*GSR*	8	30666320	A	0.025	0.000	missense			MAF
rs2253409	*GSR*	8	30666508	G	0.258	0.197	tag SNP	0.870	0.483	
rs8190997	*GSR*	8	30673453	G	0.000	0.000	missense			MAF
rs8190976	*GSR*	8	30677141	A	0.000	0.003	missense	>>	0.999	
rs8190955	*GSR*	8	30685166	T	0.000	0.000	missense			MAF
rs2978663	*GSR*	8	30685487	G	0.358		tag SNP			CALL
rs1125853	*GSR*	8	30688177	C	0.172	0.194	tag SNP	0.947	0.781	
rs8190884	*GSR*	8	30704770	A	ND		missense			CALL
rs1002149	*GSR*	8	30705280	A	0.183	0.157	3'-UTR	0.823	0.362	
rs17309872	*GSS*	20	32979449	A	0.050	0.042	5'-UTR	1.822	0.131	
rs725521	*GSS*	20	32979732	A	0.353	0.454	5'-UTR	0.781	0.097	
rs34852238	*GSS*	20	32980408	A	0.000	0.408	missense	0.976	0.875	
rs11905938	*GSS*	20	32983459	A	ND	0.000	missense			MAF
rs2236270	*GSS*	20	32986816	A	0.412	0.402	tag SNP	1.078	0.631	
rs34239729	*GSS*	20	32988287	A	0.000	0.000	missense			MAF
rs7265992	*GSS*	20	32989068	A	0.150	0.098	tag SNP	1.351	0.241	
rs6088655	*GSS*	20	32991499	A	0.376	0.455	tag SNP	0.789	0.114	
rs6060124	*GSS*	20	33000558	A	0.263	0.201	tag SNP	1.287	0.165	
rs6088660	*GSS*	20	33006557	A	0.314	0.224	tag SNP	1.294	0.152	
rs3761144	*GSS*	20	33007736	C	0.388	0.474	3'-UTR	0.806	0.144	
rs34174778	*GSS*	20	33007938	C	0.119	0.063	3'-UTR	1.829	0.056	
rs35552264	*GSS*	20	33008634	A	0.119		3'-UTR			CALL
rs13041792	*GSS*	20	33008716	A	0.217	0.235	3'-UTR	0.862	0.419	
rs2193594	*GSTZ1*	14	76855774	G	0.267	0.291	5'-UTR	1.076	0.653	
rs7160188	*GSTZ1*	14	76856114	G	0.267	0.279	5'-UTR	1.081	0.637	
rs8177539	*GSTZ1*	14	76857512	A	0.119	0.052	tag SNP	0.891	0.721	
rs3759733	*GSTZ1*	14	76858591	A	0.438	0.380	tag SNP	0.966	0.821	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs2363643	*GSTZ1*	14	76858661	A	0.283	0.323	tag SNP	1.073	0.657	
rs8177551	*GSTZ1*	14	76859876	G	0.095	0.050	tag SNP	0.842	0.609	
rs7972	*GSTZ1*	14	76862990	A	0.083	0.090	tag SNP	1.094	0.732	
rs2287396	*GSTZ1*	14	76863945	A	0.190	0.170	tag SNP	1.052	0.796	
rs1046428	*GSTZ1*	14	76864036	A	0.167	0.232	missense	1.146	0.447	
rs8004558	*GSTZ1*	14	76861793	A	0.075	0.061	tag SNP	0.673	0.209	
rs2234955	*GSTZ1*	14	76865273	C	0.000	0.000	missense			MAF
rs731346	*GSTZ1*	14	76866394	G	0.482	0.414	tag SNP	0.895	0.476	
rs8177573	*GSTZ1*	14	76866511	A	0.071	0.066	tag SNP	0.666	0.183	
rs1017186	*GSTZ1*	14	76867722	G	0.158	0.240	3’-UTR	1.133	0.470	
rs8178281	*LPO*	17	53672192	G	0.044	0.008	tag SNP	2.132	0.388	
rs8178289	*LPO*	17	53674929	C	0.168	0.212	tag SNP	0.718	0.076	
rs8178307	*LPO*	17	53678677	A	0.225	0.154	tag SNP	1.191	0.421	
rs8178318	*LPO*	17	53679987	A	0.000	0.001	missense	0.000	0.999	
rs8178338	*LPO*	17	53682931	A	0.013	0.000	missense			MAF
rs2301870	*LPO*	17	53687326	A	0.058	0.134	missense	0.762	0.203	
rs8178373	*LPO*	17	53693153	A	0.058	0.021	tag SNP	0.710	0.527	
rs8178377	*LPO*	17	53694057	A	0.053	0.057	tag SNP	1.081	0.805	
rs8178401	*LPO*	17	53698534	A	ND	0.000	missense			MAF
rs8178406	*LPO*	17	53699442	G	0.339	0.352	tag SNP	0.983	0.909	
rs8178408	*LPO*	17	53699856	C	ND	0.000	missense			MAF
rs8178412	*LPO*	17	53700313	A	0.004	0.000	missense			MAF
rs7549530	*MGST3*	1	163865270	C	0.127	0.158	5’-UTR	1.216	0.351	
rs6426925	*MGST3*	1	163865478	G	0.276	0.260	5’-UTR	1.196	0.308	
rs10737515	*MGST3*	1	163866084	A	0.490	0.468	5’-UTR	0.955	0.769	
rs6694924	*MGST3*	1	163866175	A	0.267	0.251	5’-UTR	1.162	0.392	
rs10918223	*MGST3*	1	163866273	A	0.438	0.486	5’-UTR	0.936	0.676	
rs10800118	*MGST3*	1	163866398	G	0.482		5’-UTR			CALL
rs12040685	*MGST3*	1	163866482	A	0.438	0.479	5’-UTR	0.924	0.615	
rs4147591	*MGST3*	1	163866603	A	0.267	0.256	5’-UTR	1.150	0.425	
rs4147592	*MGST3*	1	163866770	A	0.475	0.485	5’-UTR	0.970	0.845	
rs4147593	*MGST3*	1	163866891	T	0.483	0.483	5’-UTR	0.982	0.910	
rs4147594	*MGST3*	1	163867230	A	0.460	0.483	tag SNP	0.982	0.907	
rs9333372	*MGST3*	1	163867390	G	0.102	0.104	tag SNP	0.629	0.072	
rs4147597	*MGST3*	1	163869076	G	0.277	0.299	tag SNP	1.071	0.686	
rs10800120	*MGST3*	1	163871934	A	0.424	0.420	tag SNP	1.063	0.701	
rs6686643	*MGST3*	1	163883212	G	0.265	0.241	tag SNP	0.897	0.524	
rs1878076	*MGST3*	1	163883944	G	0.341	0.349	tag SNP	1.023	0.890	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs1802088	*MGST3*	1	163886899	T	ND		missense			CALL
rs11799886	*MGST3*	1	163887416	A	0.167	0.163	tag SNP	1.031	0.876	
rs4147610	*MGST3*	1	163890586	G	0.136	0.129	tag SNP	1.307	0.237	
rs7534359	*MGST3*	1	163890817	G	0.267	0.313	tag SNP	0.929	0.662	
rs8133	*MGST3*	1	163891423	T	0.438		tag SNP			CALL
rs4147611	*MGST3*	1	163891647	A	0.136	0.116	3'-UTR	1.630	0.059	
rs2759	*MPO*	17	53703105	G	0.008	0.045	missense	0.920	0.816	
rs35702888	*MPO*	17	53703207	C	0.000		missense			CALL
rs35670089	*MPO*	17	53704235	T	0.000	0.000	missense			MAF
rs7208693	*MPO*	17	53712817	A	0.040	0.081	missense	1.108	0.661	
rs2856856	*MPO*	17	53713013	G	ND		missense			CALL
rs2243828	*MPO*	17	53713883	G	0.267	0.300	3'-UTR	0.875	0.397	
rs2107545	*MPO*	17	53715117	G	0.333	0.321	3'-UTR	0.930	0.637	
rs796860	*NCF2*	1	181791478	C	0.075	0.149	tag SNP	0.874	0.525	
rs35012521	*NCF2*	1	181798987	T	0.022		missense			CALL
rs13306575	*NCF2*	1	181799060	T	0.000	0.000	missense			MAF
rs17849502	*NCF2*	1	181799203	A	0.048	0.048	missense	1.474	0.291	
rs35937854	*NCF2*	1	181802712	C	0.000	0.000	missense			MAF
rs13306581	*NCF2*	1	181802981	T	0.000	0.000	missense			MAF
rs2274064	*NCF2*	1	181809010	G	0.442		missense			CALL
rs12753665	*NCF2*	1	181812684	A	0.314	0.313	tag SNP	1.104	0.520	
rs10797888	*NCF2*	1	181813655	G	0.085	0.063	tag SNP	0.812	0.519	
rs11578964	*NCF2*	1	181822402	A	0.420	0.397	tag SNP	0.983	0.909	
rs11588654	*NCF2*	1	181823018	G	0.309	0.346	tag SNP	0.907	0.517	
rs2274065	*NCF2*	1	181826327	C	0.100	0.056	tag SNP	1.074	0.832	
rs12066019	*NCF2*	1	181828183	A	0.093	0.049	3'-UTR	1.481	0.278	
rs12096702	*NCF2*	1	181828320	G	0.121	0.049	3'-UTR	1.481	0.278	
rs12450521	*NOS2A*	17	23107519	A	0.167	0.198	5'-UTR	0.989	0.953	
rs7406657	*NOS2A*	17	23107817	C	0.181	0.211	5'-UTR	1.047	0.806	
rs28944201	*NOS2A*	17	23111317	T	0.000	0.000	missense			MAF
rs2297514	*NOS2A*	17	23117442	C	0.407		tag SNP			CALL
rs28944173	*NOS2A*	17	23117670	G	0.000	0.000	missense			MAF
rs2297518	*NOS2A*	17	23120724	A	0.167	0.170	missense	1.084	0.700	
rs11080344	*NOS2A*	17	23128638	G	0.394	0.390	tag SNP	1.052	0.751	
rs3729508	*NOS2A*	17	23133157	A	0.492	0.487	tag SNP	0.911	0.536	
rs3730017	*NOS2A*	17	23133229	T	0.008	0.000	missense			MAF
rs944725	*NOS2A*	17	23133698	A	0.375	0.342	tag SNP	1.160	0.349	
rs3794764	*NOS2A*	17	23135555	A	0.195	0.193	tag SNP	0.976	0.898	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs8072199	*NOS2A*	17	23140975	A	0.500	0.419	tag SNP	1.197	0.237	
rs3794766	*NOS2A*	17	23146048	A	0.199	0.191	tag SNP	1.213	0.323	
rs3730013	*NOS2A*	17	23150045	A	0.327	0.303	tag SNP	0.850	0.315	
rs10459953	*NOS2A*	17	23151645	C	0.457	0.000	tag SNP			MAF
rs2779248	*NOS2A*	17	23151959	G	0.300	0.356	tag SNP	0.884	0.438	
rs2779249	*NOS2A*	17	23152708	A	0.225	0.262	3'-UTR	0.891	0.494	
rs8078340	*NOS2A*	17	23153339	A	0.083	0.127	3'-UTR	0.637	0.059	
rs11857675	*NOX5*	15	67007966	G	0.050	0.057	5'-UTR	1.205	0.576	
rs16952661	*NOX5*	15	67015403	T	0.054	0.045	tag SNP	1.352	0.421	
rs3743093	*NOX5*	15	67025499	G	0.456	0.390	tag SNP	0.861	0.337	
rs311886	*NOX5*	15	67095177	A	0.050	0.056	tag SNP	1.019	0.955	
rs12443247	*NOX5*	15	67104323	A	0.100	0.099	tag SNP	0.930	0.762	
rs11072064	*NOX5*	15	67104871	A	0.322	0.280	tag SNP	0.918	0.617	
rs34097994	*NOX5*	15	67107717	G	0.000	0.000	missense			MAF
rs34406284	*NOX5*	15	67114791	A	0.000	0.000	missense			MAF
rs12907196	*NOX5*	15	67115280	G	0.458	0.432	missense	0.916	0.570	
rs2277553	*NOX5*	15	67122121	A	0.000	0.000	missense			MAF
rs2277552	*NOX5*	15	67126841	T	0.004		missense			CALL
rs7167318	*NOX5*	15	67127237	G	0.000	0.000	missense			MAF
rs7168025	*NOX5*	15	67136067	G	0.000	0.000	missense			MAF
rs35290845	*NOX5*	15	67136540	T	0.109	0.105	3'-UTR	0.802	0.386	
rs3101531	*OXR1*	8	107737708	A	0.100	0.108	5'-UTR	0.916	0.722	
rs2282509	*OXR1*	8	107739104	G	0.417	0.420	5'-UTR	1.340	0.060	
rs2282510	*OXR1*	8	107739317	A	0.409	0.441	5'-UTR	0.849	0.252	
rs1676420	*OXR1*	8	107741226	A	0.392	0.415	tag SNP	1.356	0.056	
rs1681887	*OXR1*	8	107743689	G	0.167	0.166	tag SNP	0.561	0.006	
rs3101534	*OXR1*	8	107751837	A	0.242	0.247	tag SNP	1.027	0.882	
rs17259107	*OXR1*	8	107752773	A	0.066	0.052	tag SNP	1.342	0.377	
rs28921397	*OXR1*	8	107760689	G	0.000	0.000	missense			MAF
rs1681886	*OXR1*	8	107761056	G	0.239	0.249	tag SNP	1.278	0.155	
rs3102433	*OXR1*	8	107764824	A	0.216	0.224	tag SNP	0.880	0.499	
rs10089890	*OXR1*	8	107766983	G	0.175	0.147	tag SNP	1.106	0.629	
rs3134127	*OXR1*	8	107779021	A	0.128	0.110	tag SNP	0.923	0.744	
rs1484418	*OXR1*	8	107787834	T	0.000	0.000	missense			MAF
rs28921419	*OXR1*	8	107788001	C	0.029	0.000	missense			MAF
rs28921420	*OXR1*	8	107788469	G	0.000		missense			CALL
rs3016494	*OXR1*	8	107821025	A	0.164	0.163	tag SNP	1.126	0.572	
rs28924694	*OXR1*	8	107828733	G	0.119	0.137	tag SNP	1.108	0.638	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs6983111	*OXR1*	8	107834190	A	0.242	0.244	3'-UTR	1.062	0.737	
rs9839010	*OXSR1*	3	38181910	G	0.383	0.441	5'-UTR	0.838	0.252	
rs9835496	*OXSR1*	3	38206148	T	0.083	0.126	tag SNP	0.756	0.206	
rs4955408	*OXSR1*	3	38227410	A	0.321	0.295	tag SNP	0.722	0.050	
rs272576	*OXSR1*	3	38233183	A	0.110	0.054	tag SNP	1.207	0.577	
rs1384006	*OXSR1*	3	38236041	A	0.455	0.444	tag SNP	0.803	0.152	
rs156260	*OXSR1*	3	38236811	A	0.200	0.203	tag SNP	1.121	0.542	
rs2650093	*OXSR1*	3	38238096	NA	0.000	0.000	missense			MAF
rs6599079	*OXSR1*	3	38246885	A	0.092	0.130	missense	0.743	0.176	
rs149822	*OXSR1*	3	38249538	G	0.085	0.130	tag SNP	1.144	0.531	
rs35295772	*OXSR1*	3	38264178	A	0.023	0.000	missense			MAF
rs2588458	*OXSR1*	3	38266468	NA	0.000	0.000	missense			MAF
rs4955394	*OXSR1*	3	38268467	C	0.050	0.068	tag SNP	0.870	0.619	
rs2011	*OXSR1*	3	38271821	A	0.283	0.312	tag SNP	0.924	0.639	
rs854549	*PON1*	7	94764521	A	0.381		5'-UTR			CALL
rs854550	*PON1*	7	94765178	A	0.216	0.144	3'-UTR	0.862	0.472	
rs3735590	*PON1*	7	94765431	A	0.051	0.057	tag SNP	0.603	0.138	
rs854551	*PON1*	7	94765613	A	0.203	0.147	tag SNP	0.867	0.488	
rs854552	*PON1*	7	94765860	G	0.268	0.207	tag SNP	0.799	0.217	
rs662	*PON1*	7	94775382	G	0.358	0.298	missense	0.899	0.518	
rs3917527	*PON1*	7	94778194	G	0.071	0.045	tag SNP	0.570	0.143	
rs13306698	*PON1*	7	94778718	G	0.000	0.001	missense	>>	0.999	
rs854560	*PON1*	7	94784020	T	0.397	0.401	missense	1.041	0.796	
rs705378	*PON1*	7	94784507	A	0.374	0.401	tag SNP	1.041	0.796	
rs854566	*PON1*	7	94786685	A	0.150	0.183	tag SNP	0.615	0.016	
rs705379	*PON1*	7	94791831	G	0.409		3'-UTR			CALL
rs854571	*PON1*	7	94792555	A	0.267	0.256	3'-UTR	0.874	0.451	
rs854572	*PON1*	7	94792632	C	0.441	0.393	3'-UTR	0.878	0.382	
rs854573	*PON1*	7	94792799	G	0.195	0.218	3'-UTR	0.882	0.498	
rs705382	*PON1*	7	94793157	C	0.297	0.307	3'-UTR	0.901	0.517	
rs757158	*PON1*	7	94793464	A	0.408	0.367	3'-UTR	0.891	0.451	
rs13236941	*PON1*	7	94793756	A	0.153	0.129	3'-UTR	0.953	0.829	
rs2057682	*PON3*	7	94828700	C	0.075	0.088	tag SNP	0.977	0.931	
rs17883013	*PON3*	7	94831270	A	0.009	0.000	missense			MAF
rs17878827	*PON3*	7	94834668	A	0.005	0.000	missense			MAF
rs11768074	*PON3*	7	94838527	A	0.119		tag SNP			CALL
rs2375003	*PON3*	7	94839469	T	0.000	0.000	missense			MAF
rs6977389	*PON3*	7	94857291	A	0.467	0.454	tag SNP	1.084	0.590	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs10487131	*PON3*	7	94857997	T	0.267	0.217	tag SNP	1.076	0.681	
rs10487132	*PON3*	7	94858241	G	0.447	0.450	tag SNP	0.973	0.853	
rs740264	*PON3*	7	94859739	C	0.216	0.224	tag SNP	0.946	0.753	
rs2072200	*PON3*	7	94864096	C	0.272	0.225	3'-UTR	1.079	0.664	
rs11764079	*PON3*	7	94864165	A	0.254	0.247	3'-UTR	0.963	0.830	
rs11770903	*PON3*	7	94864263	G	0.250	0.219	3'-UTR	0.981	0.914	
rs17882539	*PON3*	7	94864344	A	0.205	0.228	3'-UTR	0.964	0.833	
rs11767787	*PON3*	7	94864689	G	0.241	0.228	3'-UTR	0.964	0.833	
rs12728919	*PRDX1*	1	45748836	A	0.091	0.099	5'-UTR	0.839	0.489	
rs7903	*PRDX1*	1	45749059	C	0.442	0.489	5'-UTR	0.945	0.712	
rs1044717	*PRDX1*	1	45749174	A	0.350	0.300	5'-UTR	1.396	0.049	
rs34034070	*PRDX1*	1	45753989	G	0.024	0.000	missense			MAF
rs2356559	*PRDX1*	1	45754099	A	0.305	0.307	tag SNP	1.357	0.064	
rs12133294	*PRDX1*	1	45761351	A	0.364	0.487	3'-UTR	0.954	0.756	
rs34012472	*PRDX2*	19	12771725	G	0.009	0.000	missense			MAF
rs1205171	*PRDX2*	19	12772340	A	0.175	0.131	5'-UTR	0.958	0.833	
rs17522918	*PRDX1*	1	45760161	A	0.159	0.094	tag SNP	0.993	0.977	
rs713358	*PRDX1*	1	45760854	G	0.250		3'-UTR			CALL
rs34577345	*PRDX2*	19	12772709	A	0.000	0.000	missense			MAF
rs12151144	*PRDX2*	19	12773396	C	0.097		tag SNP			CALL
rs3377	*PRDX3*	10	120917534	A	0.370	0.493	tag SNP	1.206	0.241	
rs7768	*PRDX3*	10	120917783	C	0.250	0.317	3'-UTR	1.280	0.134	
rs36064375	*PRDX3*	10	120918744	A	0.000	0.000	missense			MAF
rs11554902	*PRDX3*	10	120921926	A	0.000	0.000	missense			MAF
rs34698541	*PRDX3*	10	120926529	C	0.000	0.000	missense			MAF
rs1553850	*PRDX3*	10	120928814	A	0.367	0.420	tag SNP	1.113	0.497	
rs3824825	*PRDX3*	10	120929061	C	0.409		3'-UTR			CALL
rs2532625	*PRDX4*	23	23594129	G	0.058	0.066	5'-UTR			NA
rs3795220	*PRDX4*	23	23594244	A	0.408	0.357	5'-UTR			HW
rs525497	*PRDX4*	23	23594917	G	0.217	0.219	5'-UTR			NA
rs513573	*PRDX4*	23	23599558	A	0.385	0.379	tag SNP			NA
rs576656	*PRDX4*	23	23601896	G	0.438	0.457	tag SNP			NA
rs499692	*PRDX4*	23	23602086	G	0.386	0.399	tag SNP			HW
rs4930698	*PRDX5*	11	63841639	C	0.114	0.080	5'-UTR	0.391	0.002	
rs28364831	*PRDX5*	11	63841724	C	0.390	0.335	5'-UTR	0.923	0.622	
rs9787810	*PRDX5*	11	63841874	A	0.410	0.335	5'-UTR	0.923	0.622	
rs11551857	*PRDX5*	11	63845667	C	0.000	0.000	tag SNP			MAF
rs1047206	*PRDX5*	11	63845792	C	0.147	0.196	3'-UTR	1.076	0.698	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs646153	*PRDX5*	11	63846164	T	0.400	0.000	3'-UTR			MAF
rs4382766	*PRDX6*	1	171711699	G	0.295	0.205	5'-UTR	1.107	0.581	
rs11576174	*PRDX6*	1	171712301	A	0.136	0.179	5'-UTR	1.168	0.450	
rs7314	*PRDX6*	1	171724222	G	0.233	0.222	tag SNP	1.077	0.692	
rs33951697	*PRDX6*	1	171724949	A	0.227	0.183	3'-UTR	1.047	0.827	
rs10306108	*PTGS1*	9	124171301	G	0.067	0.063	5'-UTR	1.102	0.744	
rs1330344	*PTGS1*	9	124171509	G	0.182	0.219	5'-UTR	0.960	0.821	
rs10306110	*PTGS1*	9	124172132	G	0.071	0.063	5'-UTR	1.102	0.744	
rs7866582	*PTGS1*	9	124178516	C	0.155	0.152	tag SNP	1.155	0.475	
rs3842789	*PTGS1*	9	124180062	A	0.000	0.000	missense			MAF
rs10306140	*PTGS1*	9	124180968	T	0.024	0.000	missense			MAF
rs10306148	*PTGS1*	9	124183205	G	0.172	0.134	tag SNP	1.257	0.286	
rs10306114	*PTGS1*	9	124172343	G	0.050	0.063	5'-UTR	1.102	0.744	
rs1236913	*PTGS1*	9	124173300	T	0.058		missense			CALL
rs3842792	*PTGS1*	9	124183528	C	0.000	0.000	missense			MAF
rs5789	*PTGS1*	9	124183794	A	0.050	0.038	missense	1.265	0.548	
rs3842798	*PTGS1*	9	124185564	G	0.250	0.176	tag SNP	1.326	0.149	
rs5791	*PTGS1*	9	124188612	G	0.000	0.000	missense			MAF
rs10985630	*PTGS1*	9	124188825	T	0.000	0.000	missense			MAF
rs12238505	*PTGS1*	9	124191705	T	0.068	0.050	tag SNP	1.294	0.443	
rs5792	*PTGS1*	9	124192328	G	0.000		missense			CALL
rs5794	*PTGS1*	9	124192442	A	0.017	0.014	missense	0.401	0.193	
rs10306194	*PTGS1*	9	124197019	A	0.142	0.193	tag SNP	1.107	0.583	
rs4648310	*PTGS2*	1	184907148	G	0.109	0.032	5'-UTR	1.962	0.137	
rs4648308	*PTGS2*	1	184907240	A	0.167	0.193	5'-UTR	0.860	0.421	
rs4648306	*PTGS2*	1	184907327	A	0.174	0.000	5'-UTR			MAF
rs689467	*PTGS2*	1	184907896	C	0.065	0.039	5'-UTR	0.535	0.119	
rs2206593	*PTGS2*	1	184909052	A	0.045	0.000	tag SNP			MAF
rs5275	*PTGS2*	1	184909681	G	0.371	0.314	tag SNP	0.885	0.450	
rs3218625	*PTGS2*	1	184910164	A	0.009	0.000	missense			MAF
rs5273	*PTGS2*	1	184910391	C	0.000	0.000	missense			MAF
rs5272	*PTGS2*	1	184910460	G	0.000	0.000	missense			MAF
rs4648279	*PTGS2*	1	184911127	C	0.000	0.000	missense			MAF
rs2066826	*PTGS2*	1	184912550	A	0.150	0.157	tag SNP	0.883	0.546	
rs3218622	*PTGS2*	1	184912628	A	0.004	0.000	missense			MAF
rs20417	*PTGS2*	1	184916944	G	0.181	0.195	3'-UTR	0.788	0.209	
rs689466	*PTGS2*	1	184917374	G	0.142	0.212	3'-UTR	0.993	0.971	
rs485902	*SCARA3*	8	27545878	G	0.455	0.447	5'-UTR	1.021	0.890	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs35775626	*SCARA3*	8	27546030	G	0.136	0.154	5'-UTR	0.926	0.712	
rs530324	*SCARA3*	8	27547105	G	0.409	0.459	5'-UTR	1.000	1.000	
rs472707	*SCARA3*	8	27547573	C	0.341	0.304	5'-UTR	1.126	0.482	
rs10503815	*SCARA3*	8	27559479	A	0.102	0.041	tag SNP	0.942	0.878	
rs17057494	*SCARA3*	8	27564204	G	0.118	0.134	tag SNP	0.898	0.633	
rs2582370	*SCARA3*	8	27564462	A	0.332	0.430	tag SNP	1.069	0.652	
rs34791518	*SCARA3*	8	27571995	A	0.000	0.011	missense	0.962	0.957	
rs17467992	*SCARA3*	8	27552600	A	0.450	0.406	tag SNP	0.969	0.839	
rs500048	*SCARA3*	8	27553709	A	0.270	0.346	tag SNP	1.112	0.488	
rs33930667	*SCARA3*	8	27572580	C	0.000	0.000	missense			MAF
rs3735754	*SCARA3*	8	27572874	T	0.009	0.000	missense			MAF
rs17057523	*SCARA3*	8	27584365	G	0.098	0.087	missense	0.975	0.925	
rs35928641	*SCARA3*	8	27584617	T	0.023		missense			CALL
rs35915165	*SCARA3*	8	27584761	A	ND	0.000	missense			MAF
rs489395	*SCARA3*	8	27584979	G	0.318	0.396	3'-UTR	1.071	0.645	
rs35590810	*SCARA3*	8	27585085	A	0.091	0.086	3'-UTR	0.906	0.732	
rs1036706	*SCARA3*	8	27585203	A	0.091	0.082	3'-UTR	0.909	0.743	
rs1036708	*SCARA3*	8	27585558	C	0.116	0.085	tag SNP	1.014	0.960	
rs518570	*SCARA3*	8	27585845	A	0.364	0.332	3'-UTR	1.048	0.766	
rs518732	*SCARA3*	8	27585906	A	0.286	0.271	3'-UTR	1.018	0.907	HW
rs485609	*SCARA3*	8	27586295	G	0.267	0.258	tag SNP	1.025	0.887	
rs1036710	*SCARA3*	8	27586394	A	0.117	0.085	tag SNP	1.014	0.960	
rs1036711	*SCARA3*	8	27586546	A	0.091	0.087	3'-UTR	0.979	0.937	
rs6413428	*SEPP1*	5	42836481	G	0.233	0.299	tag SNP	1.059	0.739	
rs28919926	*SEPP1*	5	42836623	T	0.000	0.000	missense			MAF
rs28919925	*SEPP1*	5	42836785	C	0.000	0.000	missense			MAF
rs28919923	*SEPP1*	5	42836892	A	0.000	0.000	missense			MAF
rs3877899	*SEPP1*	5	42837025	A	0.233	0.299	missense	1.059	0.739	
rs13168440	*SEPP1*	5	42839872	G	0.186	0.210	tag SNP	0.912	0.622	
rs28919895	*SEPP1*	5	42842837	T	0.013	0.000	missense			MAF
rs28919882	*SEPP1*	5	42849205	G	0.071	0.077	3'-UTR	1.473	0.193	
rs12778366	*SIRT1*	10	69313085	G	0.129	0.095	5'-UTR	1.645	0.060	
rs3758391	*SIRT1*	10	69313348	A	0.241	0.295	5'-UTR	1.069	0.680	
rs35706870	*SIRT1*	10	69313623	C	0.079	0.129	5'-UTR	0.711	0.147	
rs3740051	*SIRT1*	10	69313965	G	0.044		5'-UTR			CALL
rs932658	*SIRT1*	10	69314223	T	0.237		5'-UTR			CALL
rs2236319	*SIRT1*	10	69319226	G	0.044	0.057	tag SNP	0.923	0.810	
rs2224573	*SIRT1*	10	69335393	A	0.288	0.307	tag SNP	1.079	0.636	

SNP	Gene	Chr	Chr position	Risk allele	MAF Ref	MAF	Function	OR	P	Reason excluded
rs35224060	*SIRT1*	10	69346303	G	ND	0.000	missense			MAF
rs2234975	*SIRT1*	10	69348084	A	0.180	0.118	tag SNP	1.122	0.628	
rs2394443	*SIRT1*	10	69314347	C	0.300	0.299	5'-UTR	1.054	0.760	
rs35671182	*SIRT1*	10	69314494	A	0.000	0.000	missense			MAF
rs892034	*SIRT2*	19	44060838	A	0.466	0.458	5'-UTR	0.745	0.050	
rs2015	*SIRT2*	19	44061209	C	0.375	0.471	tag SNP	1.308	0.080	
rs34321258	*SIRT2*	19	44066146	A	ND	0.004	missense	0.511	0.586	
rs9941448	*SIRT2*	19	44070644	A	0.088	0.089	tag SNP	1.129	0.651	
rs11667030	*SIRT2*	19	44071870	A	0.345	0.444	tag SNP	1.098	0.551	
rs45535036	*SIRT2*	19	44075988	T	ND	0.007	missense	0.228	0.189	
rs10410544	*SIRT2*	19	44077372	A	0.442	0.390	tag SNP	0.894	0.456	
rs4802998	*SIRT2*	19	44079556	G	0.415	0.345	tag SNP	0.868	0.351	
rs11575003	*SIRT2*	19	44079682	G	0.077	0.079	tag SNP	1.013	0.964	
rs2053071	*SIRT2*	19	44082771	G	0.350	0.380	3'-UTR	0.974	0.863	
rs4803006	*SIRT2*	19	44083387	A	0.417	0.345	3'-UTR	0.911	0.544	
rs12979755	*SIRT2*	19	44083657	A	0.417	0.345	3'-UTR	0.911	0.544	
rs4998557	*SOD1*	21	31956763	A	0.124	0.115	tag SNP	1.195	0.436	
rs2070424	*SOD1*	21	31961191	G	0.076	0.061	tag SNP	1.249	0.463	
rs4817420	*SOD1*	21	31962242	A	0.237	0.366	tag SNP	0.996	0.981	
rs2842980	*SOD2*	6	160020106	A	0.283	0.210	5'-UTR	0.880	0.477	
rs5746141	*SOD2*	6	160022700	A	0.073	0.059	tag SNP	0.695	0.266	
rs5746138	*SOD2*	6	160022842	G	0.065	0.000	3'-UTR			MAF
rs5746136	*SOD2*	6	160023074	A	0.275	0.344	tag SNP	0.982	0.909	
rs5746129	*SOD2*	6	160025933	T	0.004	0.000	missense			MAF
rs2855116	*SOD2*	6	160026115	C	0.438	0.427	tag SNP	1.149	0.372	
rs11575993	*SOD2*	6	160029241	C	ND		missense			CALL
rs4987023	*SOD2*	6	160033683	A	0.000	0.000	missense			MAF
rs5746096	*SOD2*	6	160033880	A	ND	0.001	missense	>>	0.999	
rs2758346	*SOD2*	6	160035411	A	0.451	0.443	3'-UTR	1.076	0.631	
rs13306703	*SOD3*	4	24404611	A	0.076	0.094	5'-UTR	1.266	0.357	
rs8192287	*SOD3*	4	24405666	A	0.058	0.042	5'-UTR	1.441	0.319	
rs699473	*SOD3*	4	24405901	G	0.307	0.390	5'-UTR	1.012	0.942	
rs2536512	*SOD3*	4	24410413	A	0.383		missense			CALL
rs17879876	*SOD3*	4	24410512	NA	ND	0.000	missense			MAF
rs1799895	*SOD3*	4	24410932	G	0.033		missense			CALL
rs2855262	*SOD3*	4	24411074	A	0.383	0.388	3'-UTR	1.076	0.6386	

Legend

SNP: Single nucleotide polymorphism ID code in dbSNP.
Chr: Chromosome.
Chr position: Chromosome position according to the reference value from dbSNP.
MAF Ref: minor allele frequency in Caucasians obtained in dbSNP from HapMap data, when HapMap data were not available, any other source was accepted.
MAF: minor (risk) allele frequency.
Function: localization in the gene or reason included.

- Missense: there is a change of amino acid in the coded protein due to the presence of the SNP in the genome.
- Tag SNP: these SNPs are chosen due to their LD with many others.
- 3'-UTR: 3' region of the gene.
- 5'-UTR: 5' region of the gene.

OR: odds ratio of the association with childhood obesity adjusted by sex and age, under an additive model of inheritance.
P: significance of the association with childhood obesity adjusted by sex and age.
Reason excluded: SNPs excluded from the analysis.

- MAF: low minor allele frequency.
- CALL: call rate of the SNP under 90% of the samples.
- HW: hardy Weinberg equilibrium was not kept in the control group.

Curriculum Vitae

AZAHARA IRIS RUPÉREZ CANO

ACADEMIC EDUCATION

Sep 2009 – Dec 2010: Master's Degree in Human Nutrition, University of Granada
Sep 2005 – Jun 2007: Bachelor's Degree in Biochemistry, University of Granada
Sep 2002 – Jun 2005: Diploma of Pharmacy, University of Granada

PROFESSIONAL EXPERIENCE

Dec 2010 – Present: PhD student at the Group "CTS-461-Nutrition Biochemistry: Therapeutic Implications" in Granada, Spain.

Jul 2012 – Sep 2012 and Apr 2013 – Jun 2013: Visiting PhD student at the Division of Pediatric Endocrinology and Diabetes, in Ulm University Medical Center, Germany. Supervisor: Prof. Dr. Martin Wabitsch

Jul 2009 – Dec 2010: Full time researcher at the Group CTS-461-Nutrition Biochemistry: Therapeutic Implications" in Granada, Spain. Funded by the Network Red SAMID, RETIC RD08/0072/0028. Supervisors: Prof. Dr. Ángel Gil, Dr. Concepción M. Aguilera

Oct 2008 – Mar 2009: Internship at the Austrian Research Centers GmbH, Seibersdorf, Vienna, Austria. Funded by the ARGO internships, European Programme Leonardo da Vinci.

Sep 2007 – Jan 2008: Internship at the Institute of Biomedicine of Valencia (CSIC), Valencia, Spain. Funded by CSIC. Supervisor: Dr. Almudena Ramón Cueto.

Jul 2006 – Sep 2006: Student Internship at the Institute of Parasitology and Biomedicine López-Neyra (CSIC), Granada, Spain. Funded by CSIC. Supervisor: Dr. Alfredo Berzal Herranz.

SCIENTIFIC PUBLICATIONS

Nagel S, Keuper M, Zagotta I, Enlund E, Ruperez AI, Debatin KM, Wabitsch M, Fischer-Posovszky P. Up-regulation of Bcl-2 during adipogenesis mediates apoptosis resistance in human adipocytes. Molecular and Cellular Endocrinology 2013. (Accepted for publication on October 21st 2013)

Rupérez AI, López-Guarnido O, Gil F, Olza J, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A, Aguilera CM. Paraoxonase 1 activity and genetic variation in childhood obesity. British Journal of Nutrition 2013; 110: 1639-1647.

Rupérez AI, Olza J, Gil-Campos M, Leis R, Mesa MD, Tojo R, Cañete R, Gil A, Aguilera CM. Are Catalase -844A/G Polymorphism and Activity Associated with Childhood Obesity? Antioxidants & Redox Signaling 2013; doi: 10.1089/ars.2013.5386.

Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Influence of variants in the NPY gene on obesity and metabolic syndrome features in Spanish children. Peptides. 2013; 45: 22-27.

Olza J, Gil-Campos M, Leis R, Rupérez Al, Tojo R, Cañete R, Gil A, Aguilera CM. A gene variant of 11β-hydroxysteroid dehydrogenase type 1 is associated with obesity in children. International Journal of Obesity 2012; 36(12): 1558-1563.

PARTICIPATION IN RESEARCH PROJECTS

Association between gene variants, oxidative stress biomarkers, inflammation and cardiovascular risk in obese children (GENOBOX) (Junta de Andalucía PI11/02042).

Biological implications of insulin signalling, inflammation and extracellular matrix genes in adipose derived mesenchymal stem cells (Junta de Andalucía P10-CTS-6770).

Clinical evaluation of a dementia specific diet (Vegenat S.A.).

Evaluation of a dairy product of low energetic content and low glycemic index in obese children with non-alcoholic fatty liver disease (PRONAOS-NAFLD).

Evaluation of the glycemic index of a dairy product and its effect on apetite control (PRONAOS-INDICE GLICEMICO. Puleva Biotech S.A.).

Scientific research for the development of new generation foods for weight management and obesity prevention (CENIT-PRONAOS, CDTI).

Evaluation Of A New Orange-Based Beverage Enriched With Polyphenols (Whole Press) On Features Of Metabolic Syndrome And Cardiovascular Risk Factors In Overweight And Obese Adults Humans (Coca-Cola Services SA/SN).

Identification of single nucleotide polymorphisms of antioxidant defence system related genes and their implication in obesity and metabolic syndrome in prepubertal children (Junta de Andalucía PI-0296/2007).

STIPENDS AND AWARDS

PhD stipend from the Programa de Formación de Profesorado Universitario (FPU). From Dec 2010 to Dec 2014.

Short stay stipend from the Programa de Formación de Profesorado Universitario (FPU). From Apr 2013 to Jun 2013.

Short stay stipend from the PAP-Erasmus Programme. From Jul 2012 to Sep 2012.

Vegenat-SENPE award for best Graduate Student Poster (May 2012).

Ángel Ballabriga Stipend for Oral Communication at the XI Congress of the Spanish Society of Nutrition and Pediatric Feeding Research (SEINAP, Oct 2011).

SEMINARS, CONGRESSES AND COURSES

IUNS 20th International Congress of Nutrition (15-20 September 2013), Granada, Spain. Poster presentation: Catalase polymorphisms in childhood obesity.

VI Seminar on Healthy Diet and Novel Foods "Healthy diet and exercise, health claims and food reformulation" (3-5 July 2013), Granada, Spain.

European Congress on Obesity (ECO 2013) (12-16 May 2013), Liverpool, UK. Oral Communication: Peroxiredoxin 3 regulates adipogenic differentiation and glucose uptake in human adipocytes.

XIII Congreso de la Federación Latinoamericana de Terapia Nutricional, Nutrición Clínica y Metabolismo (FELANPE) (1-6 October 2012), Panama City.

XXVII Congreso SENPE (8-11 Mayo 2012), Madrid. Poster: Impacto del polimorfismo rs1111875 del gen HHEX (hematopoietically expressed homeobox) en el efecto de dos dietas específicas para diabéticos sobre el metabolismo glucídico.

Online course on Advanced Nutrition Statistics (Nov 2011 – Dec 2011), Sociedad Española de Nutrición Parenteral y Enteral (SENPE).

11th European Nutrition Conference (26-29 October 2011), Madrid. Poster: A variant in the PON1 gene confers protection against childhood obesity and increases PON1 lactonase activity.

XI Congreso de la Sociedad Española de Investigación en Nutrición y Alimentación Pediátrica (SEINAP) (30 Sep – 1 Oct 2011), Madrid. Oral communication: Papel de la Paraoxonasa 1 y sus variantes génicas en la protección frente a la obesidad infantile.

Data analysis workshop for massive sequencing data (Jun 2011), IT Faculty of Granada, Granada.

II Jornada científica de Jóvenes Investigadores en Biomedicina (11 de mayo de 2011), IBIMIC, Córdoba. Oral communication: Una variante del gen PON1 se asocia con la obesidad y la actividad lactonasa de la paraoxonasa 1 en niños obesos españoles.

I Simposium IDEFICS (8-9 noviembre 2010), Zaragoza. Poster: Adipocyte fatty acid-binding protein levels are increased in girls with metabolic syndrome.

Wellcome Trust Advanced Course "Genetic Analysis of Multifactorial Diseases" (21-27 Jul 2010), Wellcome Trust Centre, Hinxton, Cambridge.

Course: Protection and Animal Research for Biomedical Scientists. Category B (FELASA) (10-30 Jun 2010), Centre of Scientific Research, University of Granada.

Online course on Nutrition Statistics (Sep 2009 – Nov 2009), Sociedad Española de Nutrición Parenteral y Enteral (SENPE).

COMMUNICATIONS IN CONGRESSES

Peroxiredoxin 3 regulates adipogenic differentiation and glucose uptake in human adipocytes. Rupérez AI, Enlund E, Killian A, Aguilera CM, Wabitsch M, Fischer-Posovszky P. Obesity Facts 2013; 6 (suppl 1): 34.

Impacto del polimorfismo rs1111875 del gen HHEX (hematopoietically expressed homeobox) en el efecto de dos dietas específicas para diabéticos sobre el metabolismo glucídico. Rupérez AI, García-Rodríguez CE, Mesa García MD, Buciante G, Moreno Torres R, Pérez M, Pérez de la Cruz AJ, Gil Hernández A, Aguilera García CM. Nutricion Hospitalaria 2012; Vol 27 (suplemento 1, mayo 2012): 38.

A variant in the PON1 gene confers protection against childhood obesity and increases PON1 lactonase activity. Rupérez AI, López-Guarnido O, Olza J, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A, Gil F, Aguilera CM. Annals of Nutrition and Metabolism 2011; Vol 58(suppl 3):302.

A variant in 11β-Hydroxysteroid dehydrogenase type 1 gene is associated with obesity in children. Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Annals of Nutrition and Metabolism 2011; Vol 58(suppl 3):303.

A NPY variant is associated with obesity and traits of metabolic syndrome in Spanish children. Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Annals of Nutrition and Metabolism 2011; Vol 58(suppl 3):301.

Una variante del gen NPY se asocia con la obesidad y con características del síndrome metabólico en niños españoles. Olza J, Gil-Campos M, Leis R, Rupérez AI, Tojo R, Cañete R, Gil A, Aguilera CM. Revista española de pediatría 2011; Vol 67(5): 301.

Papel de la Paraoxonasa 1 y sus variantes génicas en la protección frente a la obesidad infantil. Rupérez AI, López-Guarnido O, Olza J, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A, Gil F, Aguilera CM. Revista española de pediatría 2011; Vol 67(5): 300.

Una variante del gen PON1 se asocia con la obesidad y la actividad lactonasa de la paraoxonasa 1 en niños obesos españoles. Rupérez AI, Olza J, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A, Aguilera CM. Libro de abstracts II Jornada de Jóvenes Investigadores del IMIBIC (ISBN: 978-84-694-3795-7) 2011;8.

Adipocyte fatty acid-binding protein plasma levels are increased in children with metabolic syndrome and correlate with non-sterified fatty acids concentration. Rupérez AI, Olza J, Gil-Campos M, Leis R, Bueno G, Tojo R, Cañete R, Moreno LA, Gil A, Aguilera CM. International Journal of Obesity 2011;35:S163.

Adipocyte fatty acid-binding protein levels are increased in girls with metabolic syndrome. Rupérez-Cano AI, Olza J, Gil-Campos M, Leis R, Bueno G, Tojo R, Cañete R, Moreno LA, Gil A y Aguilera CM. Obesity Reviews 2010;11 (Suppl. 1): 84.

Enfermedad hepática grasa no alcohólica en niños prepuberales con síndrome metabólico ¿sabemos diagnosticarla? Gil-Campos M, Martinez-Jimenez MD, Olza-Meneses O, Rupérez-Cano AI, Valle-Jimenez M, Cañete-Estrada R, Gil-Hernandez A, Aguilera-Garcia CM. Revista española de pediatría 2011; 67:40.

Variants in the KCTD15 gene identified from recent genome-wide association study are associated with obesity in Spanish children. Aguilera CM, Olza J, Rupérez AI, Gil-Campos M, Leis R, Tojo R, Cañete R y Gil A. Obesity Reviews 2010; 11 (Suppl. 1): 145.

Genomic Analysis of Tenomodulin Gene in Obese Children. Aguilera CM, Olza J, Rupérez AI, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A. J Nutrigenet Nutrigenomics 2010;3:108-109.

Variantes en el gen KCTD15 identificadas recientemente en estudios del genoma humano se asocian con obesidad en niños españoles. Olza J, Aguilera CM, Rupérez Al, Gil-Campos M, Leis R, Tojo R, Cañete R, Gil A. Revista española de pediatría 2010; Vol 66: 316.

Redesigning ribozymes. A strategy for the development of specific RNA tools. Berzal-Herranz, Alfredo; Romero-López-Martínez, Cristina; Sánchez-Luque, Francisco José; Díaz-González, Raquel; Reyes-Darias, Jose Antonio; Rupérez-Cano, Azahara Iris. Current trends in biomedicine. Workshop: RNA in disease and therapy (2006, Baeza) not published.

A new anti-hcv rna targeting the pseudoknot mortif potently blocks the ires-dependent translation. Romero-López-Martínez, Cristina; Díaz-González, Raquel; Rupérez-Cano, Azahara Iris; Berzal-Herranz, Alfredo. Current trends in biomedicine. Workshop: RNA in disease and therapy (2006, Baeza) not published.